

## Imidazolineoxyl *N*-oxide induces COX-2 in endothelial cells: role of free radicals

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### 1. ABSTRACT

cPTIO (2-[4-carboxyphenyl]-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide) exerts beneficial actions on systemic inflammatory response. Besides its nitric oxide (NO) scavenging properties cPTIO could exert beneficial effects through modulation of arachidonic acid metabolism. We studied the effect of cPTIO on the biosynthesis of vasoactive prostaglandins (PG) by endothelial cells. Human cord umbilical vein endothelial cells (HUVEC) were treated with cPTIO, and expression of cyclooxygenase (COX) isoenzymes in terms of mRNA and protein was determined by real-time-PCR and immunoblotting. Release of PGE<sub>2</sub> (as index of untransformed PGH<sub>2</sub> release) and 6-oxo-PGF<sub>1</sub>α (PGI<sub>2</sub> stable metabolite) was determined by enzyme-immunoassay. cPTIO significantly increases the release of untransformed PGH<sub>2</sub> associated to the induction of COX-2 expression. Experiments with NO-synthase inhibitors and radical scavengers showed that induction of COX-2 by cPTIO was mediated by free radical species, likely caused by the mobilization of NO from cellular stores. Finally, using specific signal-transduction inhibitors we show the involvement of Src/PI3-K/PKC pathway. Additional effects other than a direct NO scavenging activity may confer therapeutic advantages to cPTIO as compared with NO-synthase inhibitors for the treatment of systemic inflammation-associated vascular hyporeactivity.

### 2. INTRODUCTION

The nitric oxide (NO) scavenger carboxy-PTIO (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, cPTIO) exhibits a potent therapeutic value in animal models of endotoxin shock (1-6), impairs the vasorelaxation induced by acetylcholine (7), provides neuroprotection in an animal model of cerebral ischemia (8,9) and has a protective effect on toxicity caused by anthrax lethal toxin (10). cPTIO reacts with NO to yield NO<sub>2</sub> plus 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl (cPTI) (11,12). Nevertheless, emerging evidence indicates that cPTIO can exert beneficial actions on endotoxin-triggered inflammatory response either not directly due to NO scavenging (6,13,14) or independently of this ability (10). In particular, cPTIO acts on arachidonic acid (AAc) metabolism (6,13). Infusion of cPTIO significantly reduced plasma levels of LPS-induced 6-oxo-PGF<sub>1</sub>α (stable metabolite of prostaglandin (PG) I<sub>2</sub>, also called prostacyclin) and PGI-synthase (PGIS) activity (6). Results obtained from experiments performed in human cord umbilical vein endothelial cells (HUVEC) in culture and in rat aorta rings showed that PGIS inactivation caused by incubation with IL-1β plus cPTIO was probably mediated by NO<sub>2</sub>, (6,13).

Vascular endothelium plays a pivotal role in the inflammatory response regulating leukocyte recruitment, thrombosis and vascular tone. These functions are partly due to the induced prostanoid biosynthesis and release. Conversion of AAc into PGH<sub>2</sub> is catalyzed by cyclooxygenase (COX). This reaction is the first step in the biosynthetic pathways of prostanoids. *In vivo*, PGI<sub>2</sub>, PGE<sub>2</sub>, PGD<sub>2</sub>, PGF<sub>2α</sub> and thromboxane A<sub>2</sub> (TxA<sub>2</sub>) are formed from PGH<sub>2</sub> in reactions catalyzed by specific synthases (15). In previous reports, we showed that despite PGE<sub>2</sub>, PGD<sub>2</sub> and PGF<sub>2α</sub> could be detected after incubation of HUVEC with either exogenous AAc or stimuli that mobilize endogenous AAc, these prostanoids are non-enzymatic transformations of untransformed PGH<sub>2</sub> released (13,16). Release of untransformed PGH<sub>2</sub> was detected in endothelial cells in culture mainly as PGE<sub>2</sub>. In these cells PGE<sub>2</sub> is formed by non-enzymatic isomerization of the released PGH<sub>2</sub> owing to the absence of detectable expression of the inducible isoform of PGE-synthase (mPGES-1) and specific PGES activity (17). Our previous findings have recently been confirmed by Redondo and coworkers (18) who found that HUVEC express cPGES and mPGES-2, but not mPGES-1, without any detectable PGE<sub>2</sub> biosynthetic activity. The amount of untransformed PGH<sub>2</sub> released by endothelial cells depends on COX and PGIS activities, whose ratio could be modified by the induction of COX-2, the inducible isoform of COX, and by PGIS inactivation (13,16). Release of PGH<sub>2</sub> by endothelial cells has a physiological relevance since it binds to thromboxane A<sub>2</sub> receptor and exhibits vaso-constricting activity in opposition to PGI<sub>2</sub>. In addition, release of untransformed PGH<sub>2</sub> by endothelium has been observed in several physio-pathological situations (revised in (15)).

The studies described above suggest that cPTIO may exert biological actions on inflammatory syndromes characterised by strong oxidative stress not only due to NO scavenging. We previously detected inactivation of PGIS by cPTIO in biologic systems under inflammatory conditions characterized by a high rate of NO formation such as experimental endotoxin shock or exposure to IL-1β (6,13,14). The effect of cPTIO on the AAc metabolism after long exposure of endothelial cells to cPTIO is unknown. The present work therefore studies the effect of cPTIO on the COX pathway in endothelial cells.

### 3. MATERIALS AND METHODS

#### 3.1. Synthesis of cPTIO and cPTI

cPTIO and cPTI potassium salt were synthesized as previously described (6).

#### 3.2. Cell culture and treatment

Endothelial cells were isolated from human cord umbilical veins (HUVEC) and cultured as previously described (19). Cells in the first passage were seeded in 6 well plates and cultured in M199 medium containing 20% fetal bovine serum (FBS) without heparin and endothelial cell growth factor for 48 hours prior to the addition of cPTIO (0-100 microM) in M199 containing 1 % FBS. cPTIO stimulation was maintained for the indicated (in the results section) period of time until thrombin stimulation or

enzyme expression (protein or mRNA) studies were performed.

#### 3.3. Determination of arachidonic acid metabolism from endogenous substrate mobilized with thrombin

After pretreatment of HUVEC with or without cPTIO, medium was replaced and cells were incubated at 37°C with 1 U/mL thrombin for 10 minutes. Next, supernatants were removed, frozen in liquid N<sub>2</sub> and stored at -80°C until PGE<sub>2</sub> and 6-oxo-PGF<sub>1α</sub> were analyzed by specific enzyme immunoassay (EIA, GE Healthcare, Buckinghamshire, UK), following the manufacturer's instructions.

#### 3.4. COX-1 and COX-2 protein levels

After the appropriate treatments, HUVEC lysates were prepared and proteins were analyzed by immunoblotting as previously described (19,20). The effect of cPTI on COX-2 expression was analyzed incubating HUVEC with or without 100 microM of cPTI for 24 hours.

#### 3.5. COX-2 mRNA levels

Total RNA was extracted by chloroform isopropanol precipitation using Ultraspec™ (Biotecx Laboratories, Inc, Houston, Texas, USA) according to the manufacturer's instructions. Reverse transcription was performed with 1 microg of RNA per 20 microL reaction mixture and COX-2 mRNA levels were studied by real-time PCR as previously described (20). Gene expression data were normalized to β-actin as endogenous control and RNA of untreated cells was used as a calibrator sample.

#### 3.6. NO-dependence of the induction of COX-2 expression by cPTIO

HUVEC were incubated with or without 100 microM cPTIO for 24 hours, in the absence or presence of 1 mM of NO-synthase inhibitor N<sup>G</sup>-monomethyl-L-arginine (L-NMMA), 1 mM N5-[imino(nitroamino)methyl]-L-ornithine, methyl ester, (L-NAME), 120 microM of the NO-donor S-Nitroso-N-acetyl-D,L-penicillamine (SNAP, Cayman Chemical, Ann Arbor, MI), or 10 μM oxyhaemoglobin (oxyHb). Thereafter, COX-2 protein expression was evaluated as aforementioned. Oxyhaemoglobin was prepared as previously described (21). Briefly, human haemoglobin (Sigma-Aldrich Química, Madrid-Spain) was dissolved in 0.9% saline solution (100 mg/mL). Sodium dithionite was added (6.7 mg/mL) and the solution was purged with oxygen for 30 min. The solution was then centrifuged in a Microcon Centrifugal Filter Devices 10,000 MWCO (Millipore Corporation, Bedford, MA) to remove any salt. To explore the effect of reduced glutathione (GSH) and nitroso-glutathione (GSNO) on cPTIO-induced expression of COX-2 cells were incubated as mentioned above in the presence of 10 mM SGH or 1 mM GSNO (Sigma)

#### 3.7. Effect of radical scavengers on the induction of COX-2 expression by cPTIO

HUVEC were incubated with or without 100 microM cPTIO for 24 hours, in the absence or presence of 10 microL/mL DMSO, 100 microM N-acetylcysteine (NAC), 120 microM vitamin C or 100 microM vitamin E.

Thereafter, COX-2 protein expression was evaluated as described above. The effect of O<sub>2</sub><sup>-</sup> on the ability of cPTIO to induce COX-2 expression in HUVEC was evaluated by incubating cells with 100 µM cPTIO or 10 µM oxyHb for 24 hours, in the presence or absence of the indicated combinations of 500 µM xanthine sodium salt, 100 mU/mL microbial xanthine oxidase and 100 U/mL bovine superoxide dismutase–polyethylene glycol (SOD) (all compounds were purchased from Sigma). Involvement of NADPH oxidase in the action of cPTIO was investigated by incubating HUVEC with or without 100 µM cPTIO for 24 hours in the presence or absence of 30 µM diphenyleneiodonium chloride (DPI) or 1 mM of 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF).

### 3.8. Signalling pathways involved in cPTIO-induced expression of COX-2

HUVEC were incubated with 100 µM cPTIO for 24 hours, in the absence and in the presence of the indicated concentrations of GF109203X [a general inhibitor of protein kinase C (PKC)], U0126 or PD98059 [inhibitors of mitogen-activated protein kinase kinase (MEK1/2)], SB203580 [a p38 mitogen activated protein kinase (p38-MAPK) inhibitor], wortmannin or LY294002 [phosphoinoside 3-kinase (PI3-K) inhibitors], rapamycin [mammalian target of rapamycin (mTOR) inhibitor], PP-1 (4-Amino-5-(methylphenyl)-7-(*t*-butyl)pyrazolo-(3,4-*d*)pyrimidine) or PP-2 (4-Amino-3-(4-chlorophenyl)-1-(*t*-butyl)-1*H*-pyrazolo[3,4-*d*]pyrimidine) [inhibitors of Src family protein tyrosine kinases] all from Sigma; Gö6976 (a Ca<sup>2+</sup>-dependent PKCs inhibitor), and Akt-inhibitor (1*L*6-Hydroxymethyl-chiro-inositol-2(*R*)-2-*O*-methyl-3-*O*-octadecyl-*sn*-glycerocarbonate; protein kinase B inhibitor), both from Calbiochem, Darmstadt, Germany. Cells were incubated for 30 minutes with the inhibitors prior to the addition of cPTIO. Thereafter, COX-2 protein expression was evaluated as aforementioned.

To analyse activation of MAPK family, HUVEC were exposed to 100 µM cPTIO for the indicated period of time (in the Results section). Afterwards, cells were lysed and the phosphorylated MAPKs were analysed by immunoblotting, as described above for COX isoenzymes, by using polyclonal antibodies against phosphorylated p38-MAPK, ERK-1/2 and Jun N-terminal kinase (JNK; all from Promega, Madison, WI).

### 3.9. Statistics

SigmaPlot 11 software was used for statistical analysis. Statistical significance between more than two groups was assessed using one way ANOVA test, when normality test failed, one-way ANOVA on ranks was used. Student *t* test was used to compare the two groups. A "*p*" value below 0.05 was considered significant.

## 4. RESULTS AND DISCUSSION

### 4.1. Prostanoid release by HUVEC exposed to cPTIO

In Figure 1 we show the effect of cPTIO on the accumulation of prostanoids in the culture medium. cPTIO significantly increased levels of both PGI<sub>2</sub> (analysed in terms of 6-oxo-PGF<sub>1α</sub>, stable hydrolysis product of PGI<sub>2</sub>)

and PGE<sub>2</sub>. These results indicate that cPTIO was able to mobilize AAc from the membrane phosphoglycerides to be oxidized towards prostanoids. We and others have reported that PGE<sub>2</sub> is formed by non-enzymatic isomerization of PGH<sub>2</sub> outside of endothelial cells (13,16-18), since these cells do not express mPGES-1, the main isoform of PGES involved in PGE<sub>2</sub> biosynthesis (17,18). Therefore, PGE<sub>2</sub> may be representative of untransformed PGH<sub>2</sub> released by the cells.

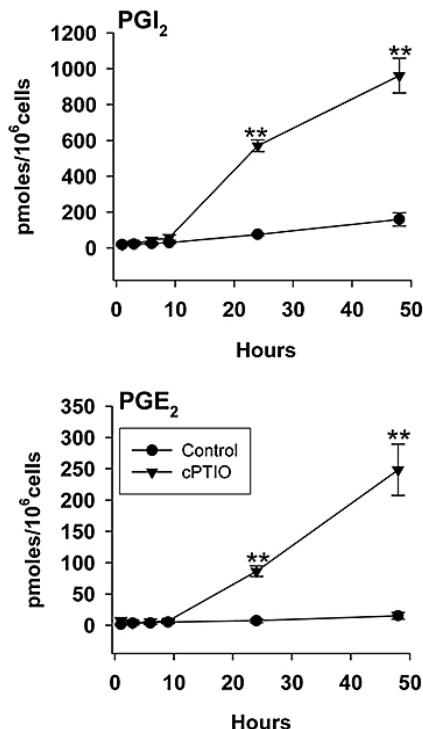
### 4.2. Effect of cPTIO on the expression of COX-isoenzymes

An excess of untransformed PGH<sub>2</sub> seems to be a consequence of COX-2 over-expression as we and others previously observed (13,16,18), thus, next we explored the effect of cPTIO on the expression of COX-2. cPTIO increased COX-2 expression in a concentration- and time-dependent manner in terms of protein (Figure 2 A and B). There was no effect of cPTIO on the expression of COX-1 (data not shown). The effect of cPTIO on COX-2 expression was at a transcriptional level since mRNA was also strongly induced in a time-dependent manner (Figure 2C), and the transcription inhibitor actinomycin-D totally suppressed the effect of cPTIO (Figure 2D). As expected, the translation inhibitor cycloheximide also inhibited cPTIO-induced expression of COX-2. To explore the effect of cPTIO on prostanoid biosynthesis in response to a phospholipase stimulus, HUVEC were incubated in the presence of several concentrations of cPTIO for 24 hours. Medium was then replaced, and HUVEC were stimulated with thrombin for 10 min to induce endogenous AAc release. Next PGE<sub>2</sub> and 6-oxo-PGF<sub>1α</sub> were measured in the cell incubation media. In these conditions cPTIO significantly increased the ability of HUVEC to produce PGE<sub>2</sub> but not 6-oxo-PGF<sub>1α</sub> (Figure 3). These findings were consistent with the up-regulation of COX-2 expression by cPTIO. The ratio PGE<sub>2</sub> to 6-oxo-PGF<sub>1α</sub> following thrombin challenge, as representative of the ratio untransformed PGH<sub>2</sub> to PGI<sub>2</sub>, increased about 17 times after 48 hours of cell exposure to 100 µM cPTIO.

Our results show that exposure of endothelial cells to cPTIO increased the ability of endothelial cells to release an excess of the vaso-constricting prostanoid PGH<sub>2</sub> when mobilization of AAc is stimulated. Release of untransformed PGH<sub>2</sub> by endothelial cells is regulated by COX-2/PGIS activity ratio (13,16). In a previous work we showed that in the presence of high amounts of NO, as a consequence of strong inflammatory conditions, cPTIO inactivates PGIS probably by the generation of NO<sub>2</sub> (6,13,14). Here, we explore the action of cPTIO on endothelial cells without any other treatment. The COX-2/PGIS activity ratio in endothelial cells is primarily increased by over-expression of the inducible COX-2.

### 4.3. Role of NO in the up-regulation of COX-2 expression induced by cPTIO

Commonly, cPTIO is merely viewed as a NO-scavenger and its biological effects are usually attributed to this property. The reaction of cPTIO with NO yields cPTI and NO<sub>2</sub> (11). We therefore investigated whether NO<sub>2</sub> or other NO derivatives mediated the cPTIO-induced COX-2



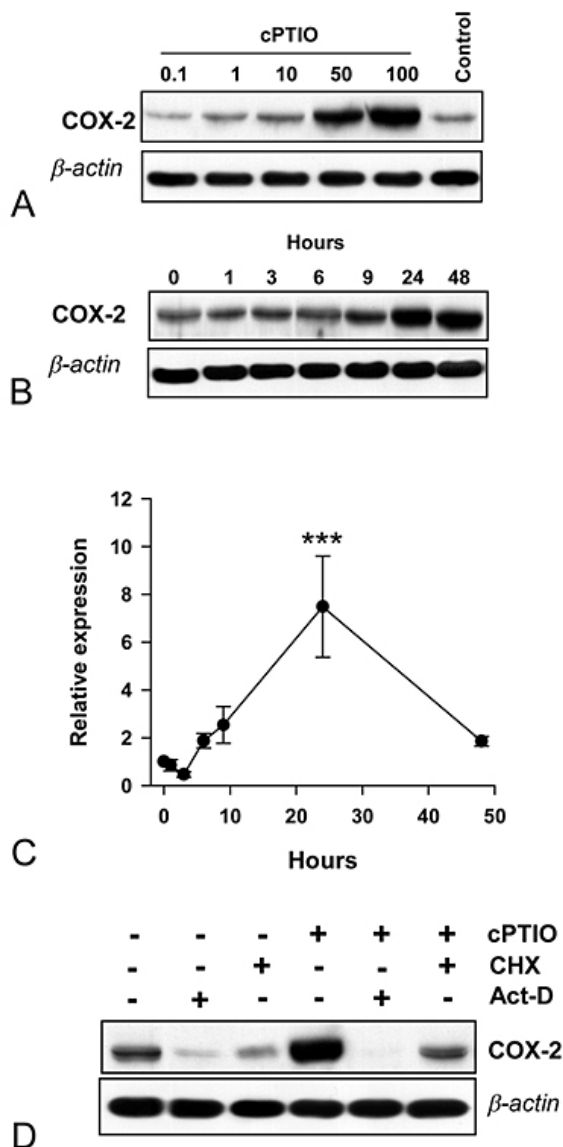
**Figure 1.** Time course of prostanoind accumulation in the culture medium as a function of the time of exposure of HUVEC to cPTIO. HUVEC were cultured with (100 microM cPTIO) or without (Control) for the indicated periods of time. PGI<sub>2</sub> (measured as its stable metabolite 6-oxo-PGF<sub>1α</sub>) and PGE<sub>2</sub> were then analyzed by EIA. Data are mean±SEM; results are expressed relative to trypan blue negative cells in each time.; n=4 \*\* p<0.01 versus controls; all comparisons refers to the same treatment time.

expression. Results in Figure 4A show that two NO-synthase inhibitors (L-NMMA and L-NAME), a NO-donor (SNAP) and cPTI (the reaction product of cPTIO with NO) did not exert any effect on COX-2 expression. The fact that cPTI was inactive in inducing COX-2 indicates that the oxygen of the N-oxide in position 3 of the imidazoline ring, which is transferred to NO to form NO<sub>2</sub> during NO-scavenging, is essential for the induction of COX-2 expression. OxyHb oxidizes NO to nitrate thereby acting as a NO-scavenger (22). We found that oxyHb also increased COX-2 expression, but in a lesser extend than cPTIO. Data in Figure 4B show that the presence of L-NMMA modified neither the effect of cPTIO nor the effect of oxyHb on COX-2 expression. Similarly, the presence of oxyHb did not modify the effect of cPTIO. The NO-donor SNAP used at low concentration attenuated the effect of cPTIO by a 47.6±3.7 % (mean±SEM, determined by densitometry, n=3) probably by transforming cPTIO to cPTI outside the cells. These results indicate that the effect of both cPTIO and oxyHb on COX-2 induction was not due to its interaction with the NO formed during the incubation period since NO synthesized *de novo* was inhibited in the presence of the NO-synthase inhibitors. The chemistry associated with the interaction of oxyHb and NO and NO-related compounds is complex. Indeed, oxyHb could reduce nitrite to generate NO, and it may also generate superoxide radical by autooxidation (23,24,25). Nevertheless, as both cPTIO and oxyHb induced COX-2

expression, this effect could be the result of the interaction of the NO-scavengers with NO from sources other than *de novo*-synthesized NO such as nitroso-derivatives. The concept that NO used by cPTIO could come from cellular stores, is consistent with our previous report (14). Indeed, we observed that the hyporesponsiveness to phenylephrine of rat aortic rings exposed to IL-1β was mainly due to NO stores formed during the incubation with the cytokine rather than those formed in the organ bath during the assay (14).

#### 4.4. Redox states of cPTIO in HUVEC

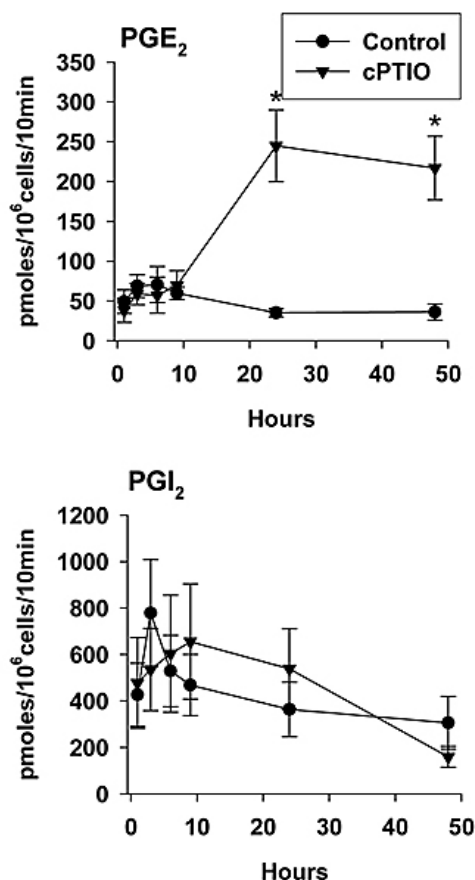
Figure 5 shows the UV-visible spectrophotometry analysis of culture medium containing cPTIO and cPTIO plus L-NMMA after 24 hours of incubation with or without cells compared with cPTI alone. No changes in the cPTI spectra were observed by the incubation with cells, either in the presence or in the absence of L-NMMA (data not shown). Data in Figure 5A show that cPTIO incubated without cells exhibited two maximum at 347 nm and 558 nm, whereas cPTI had only a maximum at 329 nm. No differences were observed between cPTIO alone and cPTIO plus L-NMMA in the absence of cells (data not shown). When cPTIO or cPTIO plus L-NMMA were incubated in the presence of HUVEC, the spectrum in the range of 300-400 nm was different than that of cPTIO without cells, and quite similar to that of cPTI. In the visible zone, spectra of cPTIO and cPTIO plus



**Figure 2.** cPTIO up-regulates COX-2 through a transcriptional mechanism. (A) Representative immunoblotting analysis of COX-2 protein expression in HUVEC as a function of cPTIO concentration (microM), HUVEC were incubated in the culture chamber at 37°C with cPTIO for 24 hours (n=3). (B) Representative immunoblotting analysis of COX-2 protein expression in HUVEC as a function of time of incubation with 100 microM cPTIO (n=3). (C) Time-course of COX-2 mRNA in response to 100 microM cPTIO; graph express data normalized to β-actin as endogenous control and relative to untreated cells as a calibrator. Mean±SEM (n=5). \*\*\*, p<0.001 vs. controls (untreated cells, time 0 hours). (D) Representative immunoblotting analysis of the effect of the transcription and translation inhibitors on cPTIO-induced expression of COX-2. HUVEC were treated or not with 100 microM of cPTIO for 24 hours in the presence or absence of 1 microM actinomycin D or 1 mg/mL cycloheximide.

L-NMMA irrespectively of the presence of cells during the incubation, were qualitatively similar to that of cPTIO alone with a maximum at 558 nm, but different than that of cPTI. When HUVEC were present during the incubation, differential spectra, subtracting cPTI spectrum, in the zone of 300-400 were similar independently of the presence of L-NMMA, but different to that of cPTIO without cells (Figure 5B, left panel). Differential spectra

analysis subtracting cPTIO shows that cPTI had a minimum at 558, whereas this valley disappeared in that of cPTIO plus L-NMMA incubated with cells (Figure 5B, right panel). The differential spectrum of cPTIO incubated with HUVEC in the absence of L-NMMA exhibited an intermediated profile between cPTI and cPTIO plus L-NMMA (Figure 5B, right panel). These results from the UV-visible spectrometric analysis were consistent with



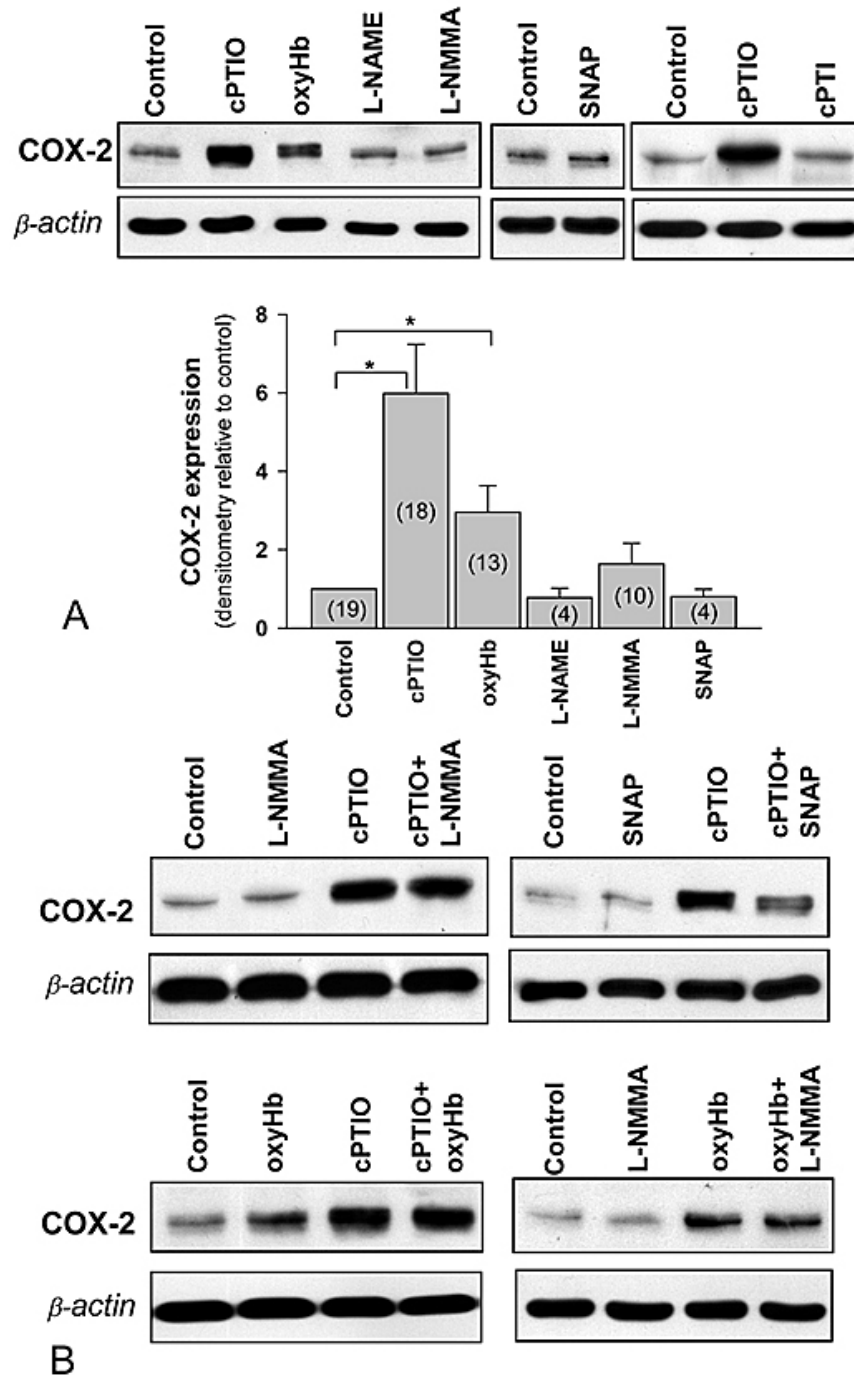
**Figure 3.** Levels of PGE<sub>2</sub> and 6-oxo-PGF<sub>1α</sub> (stable metabolite of PGI<sub>2</sub>) produced by HUVEC in response to thrombin as a function of time of exposure to cPTIO. After pretreatment of HUVEC with or without 100 microM L cPTIO for several period of time, medium was replaced and cells were incubated at 37°C with 1 U/mL thrombin for 10 minutes. In all graphs, data are the mean±SEM (n=3). \*p<0.05 vs. controls.

those reported by others (26). cPTIO could suffer reversible oxidation. Data regarding 500-600 nm indicated that cPTI was present in very small amount in cells incubated in the absence of L-NMMA, while it was apparently absent when L-NMMA was present. Our results regarding 300-400 nm, compared with spectra reported by Goldstein *et al.* (26) suggest that after incubation with HUVEC, the hydroxylamine form (c-PTIO-H) of cPTIO was present. Janssen *et al.* (27) also found that cPTIO-H was present in pulmonary cells incubated with cPTIO. Data reported by Goldstein *et al.* (26) show that cPTI, cPTIO-H and the oxoammonium cation (cPTIO<sup>+</sup>) exhibit a maximum at about 330 nm, whereas cPTIO have a maximum at about 360 nm. In addition, cPTIO<sup>+</sup> has another maximum at about 450 nm, which was not observed in our conditions.

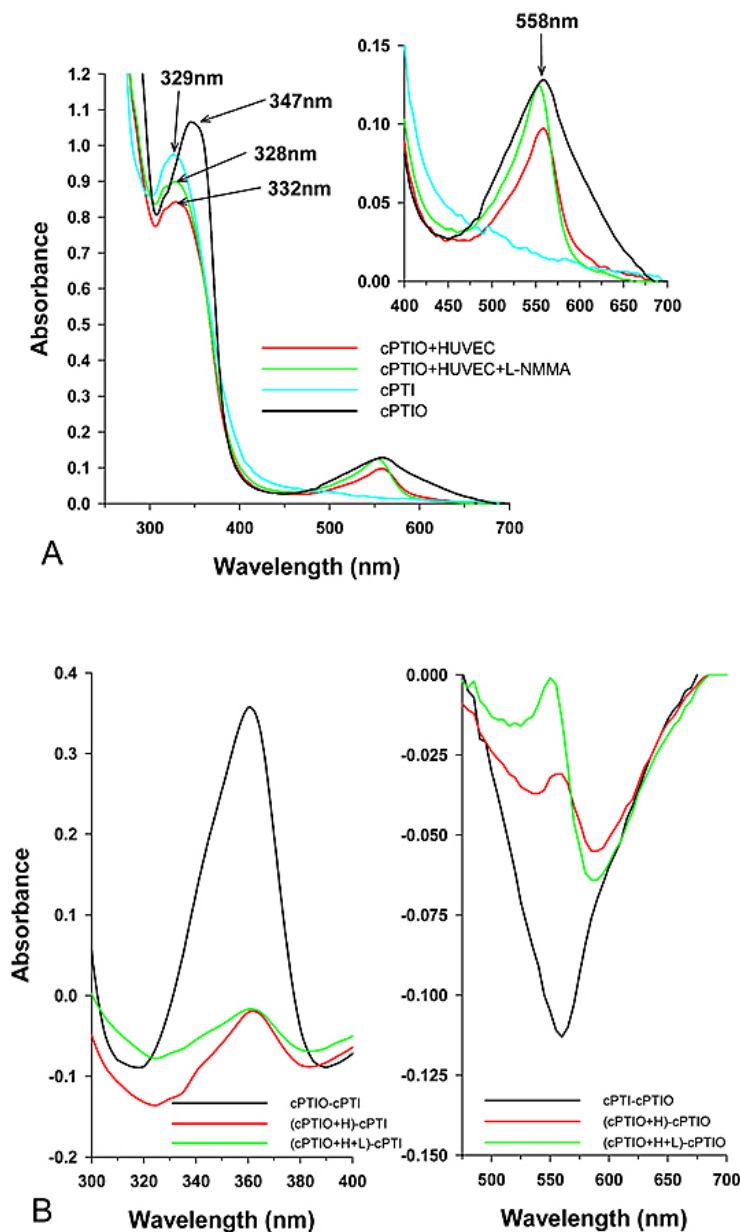
#### 4.5. Role of reactive oxygen species in the up-regulation of COX-2 by cPTIO

NO reacts rapidly with superoxide anion (O<sub>2</sub><sup>-</sup>) to yield the strong oxidant species peroxynitrite (ONOO<sup>-</sup>) (28). Endothelial cells are a relevant source of O<sub>2</sub><sup>-</sup> formed from molecular oxygen by the action of NADPH oxidases (29,30). The interaction between NO and O<sub>2</sub><sup>-</sup> regulates the

availability of NO, O<sub>2</sub><sup>-</sup> and ONOO<sup>-</sup>, NO being normally produced in excess by the endothelium (31). Depletion of endogenous NO may disrupt the balance between NO and O<sub>2</sub><sup>-</sup> and may promote O<sub>2</sub><sup>-</sup> depending responses. We then explored the effect of several radical scavengers on cPTIO-induced expression of COX-2. DMSO, NAC, vitamin C and vitamin E (Figure 6A) *per se* did not modify the expression of COX-2, whereas they significantly reduced the effect of cPTIO on COX-2 expression (Figure 6B). These data suggested that free radicals were involved in the effect of cPTIO. We then tested the effect SOD on the COX-2 induction caused by cPTIO and oxyHb (Figure 7A). SOD slightly decreased oxyHb-induced expression of COX-2, although after densitometric evaluation, difference between oxyHb with and without SOD was not statistically significant. In contrast, SOD significantly increased the effect of cPTIO on COX-2 expression by a 40.8±11.3 % (mean±SEM, determined by densitometry, n=3, p<0.05). We then tested the effect of an O<sub>2</sub><sup>-</sup> generating system on COX-2 expression. Results in Figure 7A shows that incubation with xanthine/xanthine oxidase induced COX-2 in HUVEC. Next, we explore the effect of two NADPH oxidase inhibitors on cPTIO- and oxyHb-induced



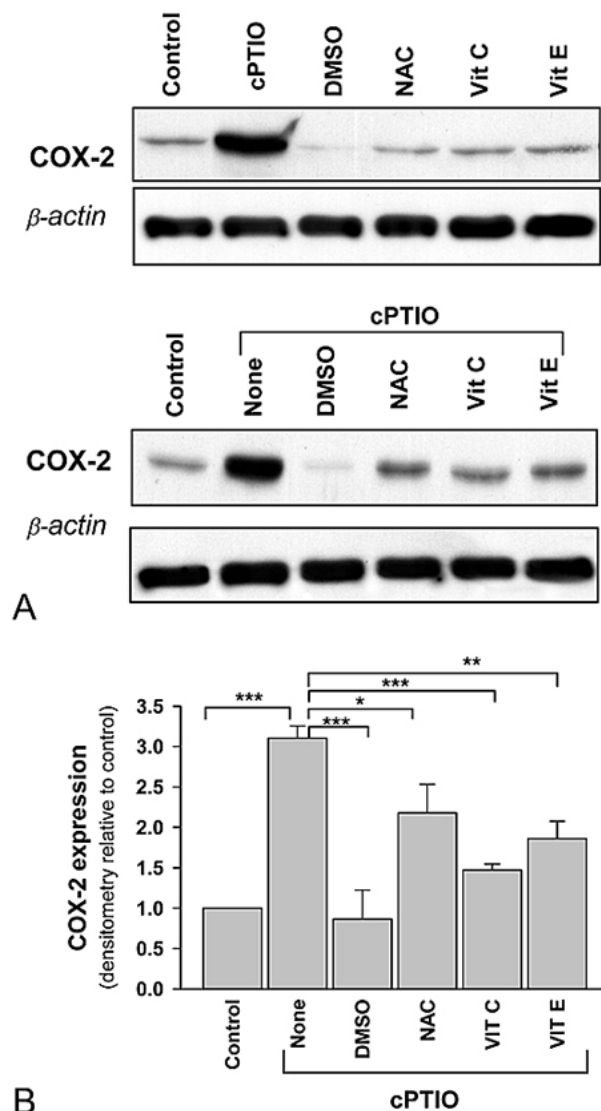
**Figure 4.** Effect of NO-scavengers and NO-synthase inhibitors on the up-regulation of COX-2. (A) Effect of different NO scavengers and NO-synthase inhibitors on COX-2 protein expression in HUVEC. Cells were incubated with 100 microM cPTIO, 10 microM L oxyHb, 1 mM of L-NMMA or L-NAME, 120 microM SNAP or 100 microM cPTI for 24 hours. Graph in panel A represents computer-assisted densitometry values normalized to the control immunoblotting band; data are the mean $\pm$ SEM, number of independent experiment is indicated in brackets; \*  $p < 0.05$ ; Kruskal-Wallis one-way ANOVA on ranks. Representative immunoblotting analysis of COX-2 are also shown. In the case of cPTI two independent experiments with identical results were performed. (B) Representative immunoblotting analysis out of three independent experiments of the effect of NO-synthase inhibitors and NO-donors on cPTIO- or oxyHb-induced expression of COX-2 in HUVEC. Cells were incubated with or without 100 microM cPTIO and/or 10 microM oxyHb for 24 hours, in the absence or presence of 1 mM of L-NMMA (NO synthase inhibitor) or 120 microM of SNAP (NO-donor). Densitometric evaluation is explained in the Results section.



**Figure 5.** UV-visible spectrometry analysis of culture medium with cPTIO after incubation with HUVEC. 100 microM cPTIO or cPTI in culture medium, free of pH indicator to avoid colour interference, was incubated alone (cPTIO, cPTI) or with HUVEC, in the presence (cPTIO+HUVEC+L-NMMA, cPTIO+H+L) or in the absence (cPTIO+HUVEC, cPTIO+H) of 1 mM of L-NMMA for 24 hours at 37°C. Culture medium was used as background. Panel A represent the UV-visible spectra and panel B depicted differential spectra.

expression of COX-2. Results in the Figure 7B show that both DPI and AEBF reduced the effect of the NO-scavengers on COX-2 expression. Collectively, these results indicated that oxygen derived free radicals were involved in the action of cPTIO and oxyHb, and that SOD could have an additional activity in the case of cPTIO. Indeed, SOD can also liberate NO from small-molecular-weight SNO (32). These results are consistent with the fact that reactive oxygen intermediates induce COX-2 expression (33). In fact, cPTIO is itself a relatively stable

oxygen-free radical that could react with free radicals other than NO, such as  $O_2^-$ . Superoxide reduced cPTIO to the corresponding hydroxylamine (cPTIO-H) which does not react with NO (26,34). It has been reported that nitroxides have SOD mimetic activity (35-37). Reaction of nitroxides with  $O_2^-$  to mimic SOD activity involves an oxoammonium/nitroxide redox couple (36,37). Oxoammonium cation (cPTIO<sup>+</sup>, in the case of cPTIO), could react with NADH or NADPH to yield NAD<sup>+</sup> or NADP<sup>+</sup> plus the corresponding hydroxylamine (cPTIO-H,



**Figure 6.** Effect of radical scavengers on the up-regulation of COX-2 by cPTIO. (A) Effect of radical scavengers on the induction of COX-2 expression. HUVEC were incubated with 100 microM cPTIO as a positive control, 10 microL/mL DMSO, 100 microM N-acetylcysteine (NAC), 120 microM vitamin C (Vit C) or 100 microM vitamin E (Vit E) for 24 hours. A representative, out of two with similar results, immunoblotting analysis of COX-2 is shown. (B) Effect of radical scavengers on the induction of COX-2 expression by cPTIO. HUVEC were incubated without (control), or with 100 microM cPTIO for 24 hours, in the absence or presence of the above mentioned scavengers. Graph in panel B represents computer-assisted densitometry values normalized to the control immunoblotting band. In the graphs, data are the mean $\pm$ -SEM (n=4). \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ; One-way repeated measures ANOVA-multiple comparisons versus control group (Bonferroni t-test).

in the case of cPTIO) (36,37). As indicated above the spectrophotometric analysis of cPTIO incubated with cells indicated that part of cPTIO was in the cPTIO-H form.

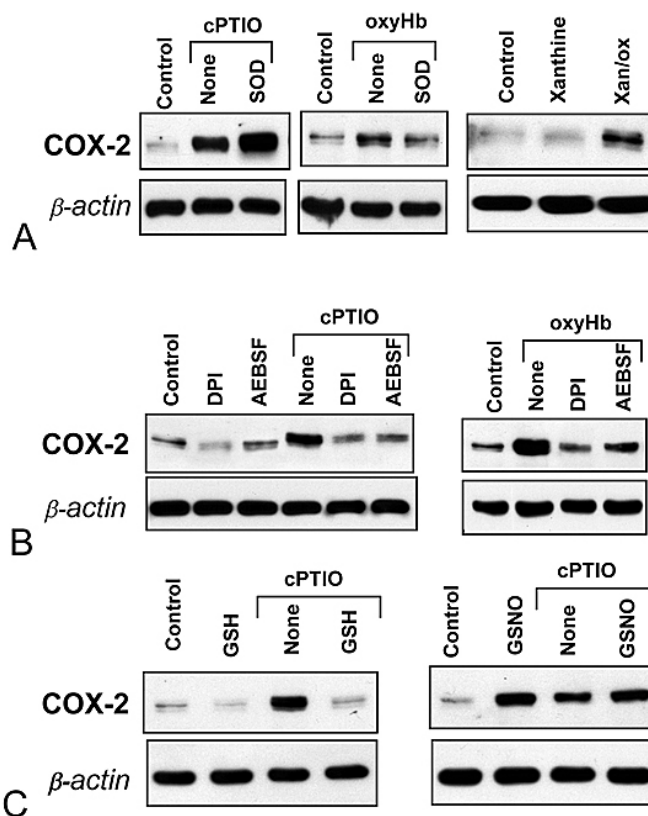
#### 4.6. PGE<sub>2</sub> released in response to cPTIO is synthesized by COX

To dismiss the possibility that PGE<sub>2</sub> and its isomers, which would be detectable by EIA, could be generated from the oxidation of AAc by free radical lipid peroxidation during the incubation with cPTIO, PGE<sub>2</sub> release was determined incubating cells with cPTIO in the

presence of 10 microM indomethacin. Indomethacin did not modify cPTIO-induced COX-2 expression and totally suppressed PGE<sub>2</sub> levels in the culture medium under any condition (data not shown). These data indicate that COX-derived PGH<sub>2</sub> was the origin of PGE<sub>2</sub> present in the extracellular medium.

#### 4.7. Generation of nitrosylating species by cPTIO

cPTIO yields NO<sub>2</sub> when reacts with NO (11), which is a nitrating species forming nitrotyrosine residues in proteins, but also can react with NO to yield the

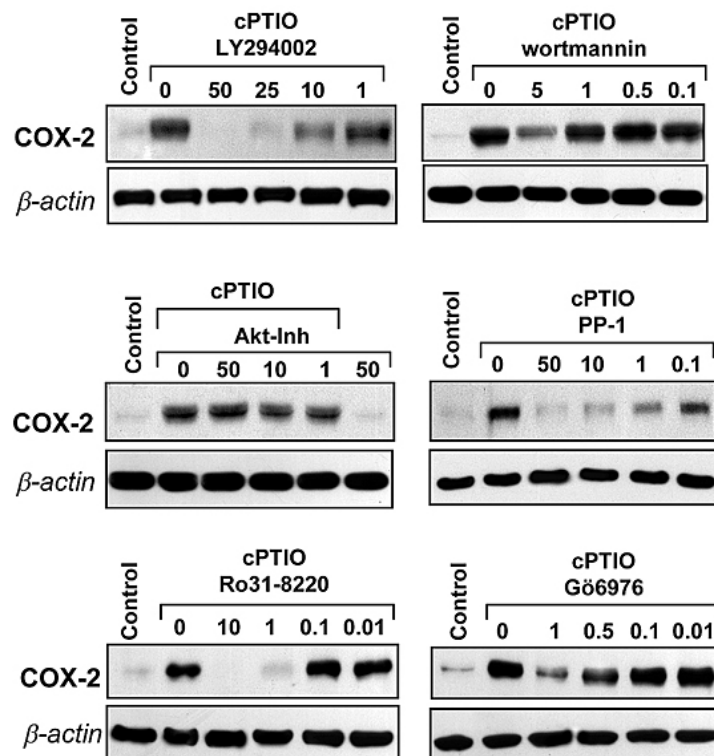


**Figure 7.** Effect of  $O_2^-$ , NADPH oxidase inhibition, GSH and GSNO on cPTIO-induced expression of COX-2 in HUVEC. Representative immunoblotting analysis out of three independent experiments are shown. (A) Cells were incubated with 10 microM oxyHb or 100 microM cPTIO in the absence or presence of 100 U/mL SOD. Cells were also incubated for 24 hours with in the presence of 500 microM xanthine (xan) with or without 100 mU/mL microbial xanthine oxidase (ox). B.- Cells were incubated with 10 microM oxyHb or 100  $\mu$ M cPTIO in the presence or absence of the 30 microM DPI or 1 mM of AEBSF (C) Cells were incubated with 100 microM cPTIO in the presence or absence of 10 mM GSH or 1 mM GSNO.

powerful thiol-nitrosating species  $N_2O_3$  (38). To explore the possibility that cPTIO could act by generating nitrosating species, we incubated cells with cPTIO in the presence of GHS, a known quencher of reactive nitrogen intermediates particularly  $N_2O_3$ . Results in Figure 7C show that GSH effectively reduced cPTIO-induced expression of COX-2. Moreover, treatment of HUVEC with 1 mM of nitroso glutathione (GSNO), a nitrosating agent (39), also induced COX-2 expression. Altogether, our results suggest that cPTIO could alter the redox state of cells. cPTIO may modify nitrosylation-denitrosylation cycle of sulfhydryls, and thereby modify cell signalling (38). A point that reinforces this concept is that cPTIO-mediated COX-2 induction was not observed in cells with a basal rate of NO biosynthesis lower than endothelial cells, such as cultured fibroblasts and vascular smooth muscle cells (data not shown). Assuming that NO could come from nitrosated proteins and/or nitrite, cPTIO could favour nitrosation and nitrosylation processes involving the mediation of  $NO_2$  (40). This, not necessarily must result in cPTI formation, since cPTIO can be regenerated if it interacts with NO in the oxidized oxoammonium form (26).

#### 4.8. Signalling pathways involved in the cPTIO-induced expression of COX-2

To characterize signalling pathways involved in the cPTIO-induced COX-2 expression, cells were treated with several inhibitors during cPTIO stimulation. As a first approximation, we analyzed COX-2 expression in cells stimulated with cPTIO in the presence of a constant concentration of inhibitor, as follows: Ro31-3220 (10  $\mu$ M), GF109203X (5  $\mu$ M), Gö6976 (1  $\mu$ M), rapamycin (1 microM), SB203580 (10  $\mu$ M), U0126 (10  $\mu$ M), PD98059 (50  $\mu$ M), LY294002 (50 microM), Akt-inhibitor (25 microM) and PP-1 and PP-2 (10 microM). Densitometric evaluation of the immunoblottings showed that the PI3-K inhibitor LY294002 significantly inhibited the effect of cPTIO by a percentage of  $99.2 \pm 6.8$  ( $n=3$ ,  $p<0.05$  when compared with cPTIO sample without inhibitor), whereas the Akt-inhibitor did not modify COX-2 expression at all. The PKC general inhibitors Ro31-3220 and GF109203X significantly inhibited the effect of cPTIO by a percentage of  $100 \pm 0.0$  (mean  $\pm$  SEM) ( $n=2$ ,  $p<0.05$ ) and  $45.4 \pm 2.0$  ( $n=3$ ,  $p<0.01$ ), respectively. The  $Ca^{++}$ -dependent PKC inhibitor Gö6976 also significantly inhibited the cPTIO effect by a percentage of  $61.0 \pm 2.6$  ( $n=3$ ,  $p=0.01$ ). The Src family protein tyrosine kinase inhibitors PP-1 and PP-2 at



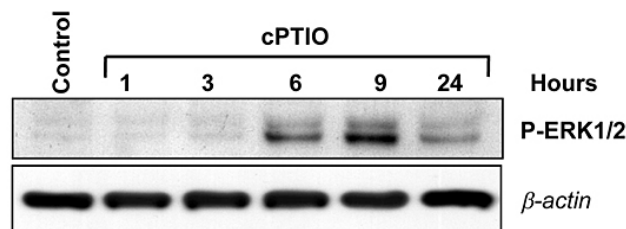
**Figure 8.** Signalling pathways involved in the up-regulation of COX-2 by cPTIO. Representative, out of two with similar results, immunoblotting analysis of the concentration-dependent effect of PI3-K, PKC, Src signalling pathways inhibitors on cPTIO-induced expression of COX-2. HUVEC were incubated without (control) or with 100 microM cPTIO for 24 hours, in the presence of the indicated concentrations (microM) of the inhibitors: LY294002, wortmannin, Akt-inhibitor (Akt-inh), PP-1, Ro31-8220 and G66976.

concentration of 10 microM partially abrogated cPTIO-induced COX-2 expression by a 39.5 $\pm$ 6.8 and 41.8 $\pm$ 4.3 % respectively (mean $\pm$ SEM, n=3, p<0.05). The MEK1/2 inhibitors U0126 and PD98059, the p38 MAPK inhibitor SB203580 and the mTOR inhibitor rapamycin, did not exert a significant inhibition of COX-2 expression induced by cPTIO.

Since the above results suggested that PI3-K was involved in the cPTIO-induced COX-2, further experiments were carried out to observe the concentration-dependent effect of two PI3K inhibitors (LY294002 and wortmannin) and therefore rule-out the possibility that the effects observed in the preliminary experiments were unspecific. Figure 8 depict representative western-blot showing that both inhibitors suppressed the effect of cPTIO on the COX-2 protein expression in a concentration-dependent manner. Since Akt is a canonical downstream effector of PI3-K, we also tested the effect of Akt-inhibitor on cPTIO-induced COX-2 expression. However, results in Figure 8 clearly show that 50  $\mu$ M of Akt-inhibitor *per se* neither modify the expression of COX-2, nor the induction of COX-2 by cPTIO. Our results are consistent with the fact that PI3-K has been described as a critical link in signalling of reactive oxygen species (41). These results also rule out the involvement of PI3-K/Akt pathway in the induction of COX-2 by cPTIO.

PKC isoforms have been classified into three groups according to their structure and cofactor requirements: conventional PKCs calcium- and DAG-dependent, novel PKCs DAG-dependent and calcium independent and atypical PKCs calcium independent, which are not activated by phorbol esters. PI3-K could also regulate conventional PKC (42). Consistently we confirmed that the general PKC inhibitor Ro31-3220 and the calcium-dependent PKC inhibitor G66976 reduced cPTIO-induced expression of COX-2 in a concentration-dependent manner (Figure 8). The inhibition of cPTIO-induced expression of COX-2 by a calcium-dependent PKC inhibitor suggests the particular implication of this class of PKCs in the action of cPTIO. Taken together, these results suggest that PI3-K/PKC is involved in cPTIO-induced COX-2 expression. Direct PKC activation by oxidant species has also been described (43), which should be consistent with the involvement of oxygen-derived reactive species such as O<sub>2</sub><sup>-</sup> on cPTIO-induced COX-2 expression.

The Src family protein tyrosine kinase inhibitor PP-1 also concentration-dependently suppressed the effect of cPTIO on the COX-2 protein expression (Figure 8). Src protein tyrosine kinases are non receptor tyrosine kinases that initiate a sequential phosphorylation through MAPK pathway that includes Ras, Raf/MEK and ERK1/2 and can also activate Ras/PI3-K pathway. Src and Ras can be activated either by nitrosylation or by O<sub>2</sub><sup>-</sup> (44-47).



**Figure 9.** Representative immunoblotting analysis of phosphorylated ERK1/2 in HUVEC as a function of time of exposition to 100 microM of cPTIO (n=3).

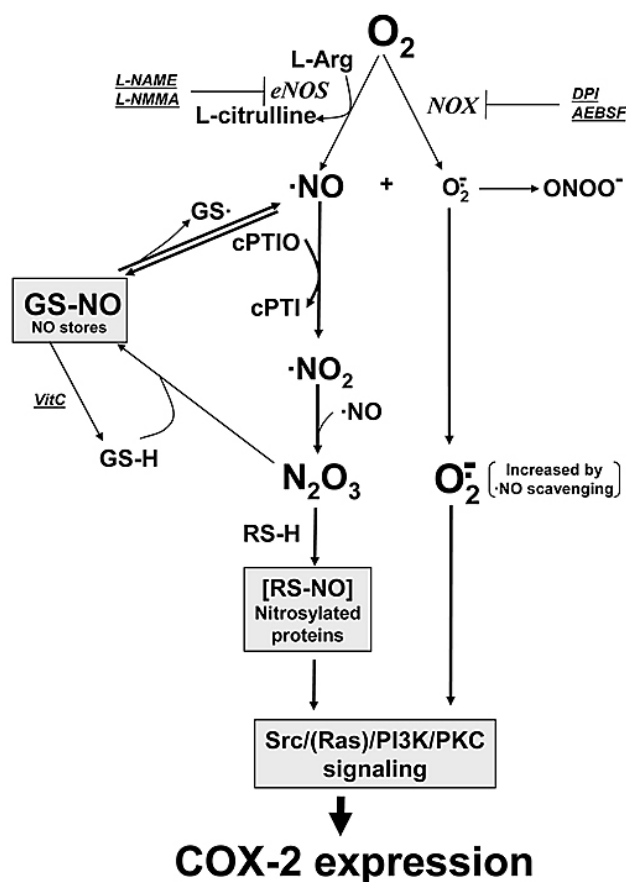
Three subfamilies of MAPKs, ERK, JNK and p38-MAPK, play pivotal roles in a variety of cellular functions and they are involved in the regulation of many genes. To further evaluate the involvement of the MAPK family in the induction of COX-2 by cPTIO we examined phosphorylation of p38-MAPK, JNK and ERK1/2 after treatment of HUVEC with cPTIO for several periods of time. Anti-phospho-MAPKs antibodies were used to perform immunoblotting analysis. No effect was observed in response to cPTIO regarding either JNK or p38-MAPK phosphorylation in the incubation period ranging from 10 min to 48 hours (data not shown). In contrast, cPTIO triggered ERK1/2 phosphorylation in a time dependent manner, although this was observed 6 hours after the addition of cPTIO to the cells (Figure 9). ERK1/2 phosphorylation usually occurs within minutes after cell challenge, but cPTIO induced a late phosphorylation. One possible explanation for this finding could be that the phosphorylation of ERK1/2 was secondary to the cPTIO-mediated induction of other effector/s. The fact that neither actinomycin D, (a transcription inhibitor), nor cycloheximide (a translation inhibitor) suppressed ERK1/2 phosphorylation (data not shown), rules out the possibility that this putative secondary effector was a protein transcriptionally regulated by cPTIO. In order to establish if phosphorylation of ERK1/2 mediated cPTIO-induced expression of COX-2, we tested whether the transduction signal inhibitors related with the pathways involved in the cPTIO-induced expression of COX-2, Ro31-3220, Gö6976, LY294002 and Akt-inhibitor, inhibited ERK1/2 phosphorylation. We observed that not only none of them attenuated ERK1/2 phosphorylation but that they even increased this, indicating that this MAPK was not involved in the up-regulation of COX-2 expression by cPTIO in HUVEC (not shown). Phosphorylation of ERK1/2 is nevertheless consistent with the activation of Src/Ras pathway caused by cPTIO (48).

We previously reported that cPTIO could act indirectly on the metabolism of AAc inhibiting PGIS activity, likely throughout the generation of NO<sub>2</sub> (6,13,14). Herein, we show that cPTIO induces COX-2 expression. Together these actions result in the release of untransformed PGH<sub>2</sub> by endothelial cells, suggesting it could contribute to the positive vasopressor activity of cPTIO (2,4,7) irrespectively of its action as an NO scavenger. This could be particularly relevant for systemic inflammatory responses characterized by a vascular hyporeactivity such as endotoxic shock (1-6), since cPTIO

could add a therapeutic value compared with NO-synthase inhibitors. Additionally, our results show that caution must to be taken into account when cPTIO is used to ascertain the role of NO in a particular physiopathologic process, since cPTIO could exert diverse effects. Indeed, cPTIO was able to trigger signalling pathways that could potentially influence gene expression, cell apoptosis or cell cycle progression. This wide range of biological effects of cPTIO could account for a number of non-expected observations in experimental approaches using this drug (9,10,49).

In our previous work (6) we show that cPTIO improved blood pressure and mortality in an experimental shock model by inhibition of PGI-synthase in addition to NO scavenging. This leads to a reduced formation of the potent vasodilator PGI<sub>2</sub>. We also have shown that the presence of cPTIO in the organ bath, added after the treatment of aortic rings with IL-1β, improved contractility more than L-NAME. The hyporeactivity of rat aorta exposed to IL-1β was mainly due to pre-formed NO-stores rather to a NO formed during the stimulation in the organ bath (14). Our current results indicate that cPTIO could induce COX-2 expression in the vascular endothelium resulting in the release of the potent vaso-constrictor prostaglandin PGH<sub>2</sub>. All these additional effects may confer therapeutic advantages to cPTIO as compared with NO-synthase inhibitors for the treatment of systemic inflammation-associated vascular hyporeactivity.

In conclusion, in this study we found that in HUVEC cPTIO significantly increases the release of untransformed PGH<sub>2</sub> (measured as PGE<sub>2</sub>) associated to a time- and concentration-dependent induction of COX-2 expression. The induction of COX-2 by cPTIO was mediated by free radical-species. Although we have not identified the molecular species and the exact mechanism involved in cPTIO induced expression of COX-2 expression, taken together, our results strongly suggest that this effect was dependent of the reaction with NO coming from intracellular pools probably nitroso-derivatives. NO quenching by cPTIO produces as outcome an increased availability of superoxide and generation of NO<sub>2</sub>, which could result in the generation of the nitrosating agent N<sub>2</sub>O<sub>3</sub>. These facts could lead to an activation of Src/PI3-K/PKC pathway through nitrosylation and/or directly mediated by superoxide (this is schematized in Figure 10). More research is needed to establish the most relevant way leading COX-2 expression.



**Figure 10.** Putative mechanism of action of cPTIO. NO mainly coming from cellular stores is quenched by cPTIO which increase the availability of superoxide and also generates NO<sub>2</sub>, which could result in the generation of the nitrosating agent N<sub>2</sub>O<sub>3</sub>. These facts lead to an activation of Src/PI3-K/PKC pathway through nitrosylation and/or directly mediated by O<sub>2</sub><sup>-</sup>.

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- Abbreviations:** AAc: arachidonic acid, COX: cyclooxygenase, AEBFS: 4-(-2-aminoethyl)-benzenesulfonyl fluoride, cPGES: cytosolic PGE-synthase, cPTI: 2-(4-carboxyphenyl)-4:4:5:5-tetramethylimidazoline-1-oxyl, cPTIO (2-[4-carboxyphenyl]-4:4:5:5-tetramethylimidazoline-1-oxyl-3-oxide, DPI: Diphenyliodonium chloride, FBS: fetal bovine serum, HUVEC: umbilical vein endothelial cells, GSH: reduced glutathione, GSNO: nitroso-glutathione, JNK: Jun N-terminal kinase, L-NAME: N<sup>G</sup>-[imino(nitroamino)methyl]-L-ornithine: methyl ester, L-NMMA: N<sup>G</sup>-monomethyl-L-arginine, mPGES-1: microsomal PGE-synthase-1, MEK1/2: mitogen-activated protein kinase kinase-1 and 2, mTOR, mammalian target of rapamycin, NAC: N-acetylcysteine, NO: nitric oxide, oxyHb: oxyhaemoglobin, p38-MAPK: p38 mitogen activated protein kinase, PG:prostaglandin, PGES: PGE-

## Cptio induces COX-2 in human endothelial cells

synthase, PGIS: PGI-synthase, PI3-K: phosphoinoside 3-kinase, PKC: protein kinase C, PP1: 4-Amino-5-(methylphenyl)-7-(t-butyl)pyrazolo-(3:4-d)pyrimidine, PP2: 4-Amino-3-(4-chlorophenyl)-1-(t-butyl)-1H-pyrazolo[3:4-d]pyrimidine, SNAP: S-Nitroso-N-acetyl-D:L-penicillamine, TxA<sub>2</sub>: thromboxane A<sub>2</sub>

**Key Words:** cPTIO, COX-2, Endothelial cell, Nitric oxide, PI3K, PKC, Src

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