

Extracellular histones disarrange vasoactive mediators release through a COX-NOS interaction in human endothelial cells

Daniel Pérez-Cremades^{a, b}, Carlos Bueno-Betí^{a, b}, José Luis García-Giménez^{a, b, c}, José Santiago Ibañez-Cabellos^{a, b, c}, Carlos Hermenegildo^{a, b}, Federico V. Pallardó^{a, b, c, *}, Susana Novella^{a, b, *}

^a *Department of Physiology, Faculty of Medicine and Dentistry, University of Valencia, Valencia, Spain*

^b *INCLIVA Biomedical Research Institute, Valencia, Spain*

^c *Center for Biomedical Network Research on Rare Diseases (CIBERER), Institute of Health Carlos III, Valencia, Spain*

Received: September 23, 2016; Accepted: December 13, 2016

Abstract

Extracellular histones are mediators of inflammation, tissue injury and organ dysfunction. Interactions between circulating histones and vascular endothelial cells are key events in histone-mediated pathologies. Our aim was to investigate the implication of extracellular histones in the production of the major vasoactive compounds released by human endothelial cells (HUVECs), prostanoids and nitric oxide (NO). HUVEC exposed to increasing concentrations of histones (0.001 to 100 µg/ml) for 4 hrs induced prostacyclin (PGI₂) production in a dose-dependent manner and decreased thromboxane A₂ (TXA₂) release at 100 µg/ml. Extracellular histones raised cyclooxygenase-2 (COX-2) and prostacyclin synthase (PGIS) mRNA and protein expression, decreased COX-1 mRNA levels and did not change thromboxane A₂ synthase (TXAS) expression. Moreover, extracellular histones decreased both, eNOS expression and NO production in HUVEC. The impaired NO production was related to COX-2 activity and superoxide production since was reversed after celecoxib (10 µmol/l) and tempol (100 µmol/l) treatments, respectively. In conclusion, our findings suggest that extracellular histones stimulate the release of endothelial-dependent mediators through an up-regulation in COX-2-PGIS-PGI₂ pathway which involves a COX-2-dependent superoxide production that decreases the activity of eNOS and the NO production. These effects may contribute to the endothelial cell dysfunction observed in histone-mediated pathologies.

Keywords: extracellular histones • endothelial cells • vascular mediators • nitric oxide • prostanoids

Introduction

Recent studies indicate that histones mediate proinflammatory activity when are released into extracellular space [1]. In this regard, high levels of circulating histones in plasma have been detected in trauma-associated injury [2], ischaemia–reperfusion injuries in kidney [3] liver [4] and sepsis [5, 6].

Endothelium participates in numerous regulatory functions and contributes to and is affected by inflammatory processes. It is also involved in blood coagulation and fibrinolysis, immune response by modulation of leucocyte interactions with the vessel wall and regulation of vascular tone and blood pressure [7]. Disturbance of the endothelium functional integrity in response to circulating compounds reflects a first step in many disorders. After pro-inflammatory stimuli, endothelium undergoes activation characterized by increased

local blood flow, leakage of plasma-protein-rich fluid into the tissues and recruitment and activation of circulating leucocytes [8].

Endothelium exerts these actions through the release of vasoactive compounds, including prostanoids and nitric oxide (NO), that control functions of both vascular smooth muscle cells and of circulating blood cells [7].

Cyclooxygenases (COX) are the rate-limiting enzymes in the production. COX isoenzyme (COX-1 and COX-2) expression has been detected in the vascular system [9]. COX-1 is considered the constitutive isoform in endothelium, while COX-2 is induced under pro-inflammatory conditions. However, both COX share characteristics of constitutive and inducible enzymes in endothelial cells [10]. On the other hand, NO is mainly produced by endothelial nitric oxide

*Correspondence to: Dr. Federico V. PALLARDÓ
E-mail: Federico.v.pallardo@uv.es
and

Dr. Susana NOVELLA
E-mail: Susana.novella@uv.es

synthase (eNOS) in the vasculature [11]. NO dilates blood vessels through acting on smooth muscle cells and inhibits platelet aggregation. NO also decreases leucocyte adhesion by suppressing cell adhesion molecule expression on cell membrane surface, therefore contributing to quiescence of resting endothelial cells [12].

Endothelial cells exposed to extracellular histones release pro-inflammatory cytokines, induce tissue factor expression [13] and increase adhesion molecules in the cell membrane [14]. However, the vasoactive effect of extracellular histones has been less studied. Therefore, the aim of this study was to describe the implication of extracellular histones in the release of the two major vasoactive compounds released by endothelial cells, NO and prostanoids.

Materials and methods

Cell culture and experimental design

Primary human umbilical vein endothelial cell (HUVEC) cultures were obtained from human umbilical cord from 'Hospital Clínico Universitario' of Valencia as previously described [15]. Briefly, umbilical veins were treated with 1% collagenase (Life Technologies, Carlsbad, CA, USA), and HUVECs extracted were cultured in specific endothelial growth medium, EGM-2 (Lonza, Cultek, Barcelona, Spain).

HUVECs from passages 3 to 5 were used in this study. When they reached confluence, media were changed and cells were exposed during 4 hrs to different calf thymus (CT) histone concentrations (Sigma-Aldrich, St. Louis, MO, USA): 1, 10, 100 ng/ml and 1, 10, 25, 50 and 100 µg/ml prepared in PBS and free of LPS. In some experiments, 10 µmol/l celecoxib, a specific COX-2 inhibitor (Sigma-Aldrich) and 100 µmol/l tempol (Sigma-Aldrich), a superoxide dismutase mimetic, were added to HUVEC 1 hr before histone treatments.

Cells were identified as endothelial by their characteristic cobblestone morphology and the presence of von Willebrand factor by immunofluorescence using a specific antibody (ab6994; Abcam, Cambridge, UK). Cells used in this study were more than 95% vWF positive.

Cell viability was measured by flow cytometry using propidium iodide (Immunostep, Salamanca, Spain). Our results showed that extracellular histones scarcely affect HUVEC viability. Only at higher concentrations, extracellular histones induce a mortality of 6.4% (50 µg/ml) and 10.9% (100 µg/ml) respect to non-treated cells.

The investigation conforms to the principles outlined in the Declaration of Helsinki and was approved by the Ethical Committee of Clinical Research of the INCLIVA, 'Hospital Clínico Universitario' of Valencia, and written informed consent was obtained from all donors.

Nitric oxide measurement

NO production was determined by fluorescence microscopy and 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM Diacetate) (Life Technologies, Alcobendas, Madrid, Spain) probe. After 3-hrs treatment, DAF-FM was added to culture media and incubated for 45 min. Media were then replaced, and cells were incubated for 15 additional minutes to ensure complete fluorescence probe deacetylation.

NO production was determined by measuring intensity fluorescence at 515 nm on an inverted fluorescence microscope (Eclipse Ti-S; Nikon

Co, Tokyo, Japan). Three randomly selected pictures per condition tested were taken, and fluorescence intensity measurements were recorded using NIS-Elements 3.2 software (Nikon Co). Results are presented as the mean intensity fluorescence per power field subtracting the background and were relativized to non-treated cells.

Prostacyclin and thromboxane A2 determination

The amount of PGI₂ and TXA₂ produced was measured by enzyme immune assay using commercial EIA kits (Cayman Chemical, Ann Arbor, MI, USA) as previously described [16]. After treatments, media were collected and stored at -80°C. Cells were lysed in RIPA buffer (Sigma-Aldrich) for protein determination, calculated by the Pierce BCA protein assay kit using BSA as a standard (Thermo Scientific Inc., Rockford, USA).

PGI₂ and TXA₂ levels were calculated as the concentration of stable hydrolysis metabolite products, 6-keto-prostaglandin-F1alpha and TXB₂, respectively. Results were expressed as the ratio increases over untreated control in ng prostanoid/mg protein.

RNA isolation and quantitative real-time PCR assay (qRT-PCR)

Total RNA was extracted using TRIzol reagent (Invitrogen, Barcelona, Spain) following the manufacturer's instructions. Reverse transcription of 200 ng of total RNA was carried out using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) using Mastercycler Eppendorf Thermocycler (Eppendorf, Hamburg, Germany). The mRNA levels were determined by qRT-PCR analysis using an ABI Prism 7900 HT Fast Real-Time PCR System (Applied Biosystems). Gene-specific primer pairs and probes were purchased from Thermo Fisher (Assay-on-Demand) for eNOS (Hs01574659_m1), COX-1 (Hs00377726_m1), COX-2 (Hs00153133_m1), PGIS (Hs00919949_m1) TXAS (Hs00233423_m1) and GAPDH (endogenous control, Hs99999905_m1) and were used with TaqMan Universal Mastermix (Thermo Fisher, Rockford, IL, USA). PCR conditions were 10 min. at 95°C for enzyme activation, followed by 40 two-step cycles (15 sec. at 95°C; 1 min. at 60°C). Data were analysed with the SDS 2.2.2 software (Applied Biosystems) according to the 2^{-ΔΔC_T} method.

Western blot

Treated HUVECs were collected in RIPA buffer (Sigma-Aldrich) containing protease and phosphatase inhibitors (Roche Diagnostics, Madrid, Spain). Protein content was measured by the Pierce BCA protein assay kit (Thermo Scientific Inc.) using BSA as a standard. Equal amounts of protein were then separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Whatman, GE Healthcare Life Sciences, Chicago, IL, USA). Immunostaining was achieved using specific antibodies: eNOS (sc-653), COX-1 (sc-19998), COX-2 (sc-19999), PGIS (sc-20933), TXAS (sc-79181) all from Santa Cruz BioTechnology (Heidelberg, Germany) and β-actin (Sigma-Aldrich) as loading control. Development was performed peroxidase-linked secondary antibodies (Santa Cruz Biotechnology). Luminol (ECL Western Blotting Detection Reagents, GE Healthcare, Hatfield, and Hertfordshire, UK) was added onto the membrane, and membranes were revealed by an image reader LAS-4000 (GE Healthcare, Uppsala, Sweden). Signal density was analysed with ImageJ software (NIH Image, National Institutes of Health, Bethesda, MD, USA).

Superoxide determination

Intracellular superoxide concentration was detected by measuring dihydroethidium (DHE) oxidation. DHE enters in the cell and is oxidized by superoxide to yield ethidium. Binding of ethidium to DNA produces red fluorescence. Histone-treated cells were loaded with 2.5 $\mu\text{mol/l}$ DHE for 30 min. Then, cells were rinsed with PBS and observed under an inverted fluorescence Nikon Eclipse Ti-S microscope. Fluorescence from three different fields per well was measured (excitation wavelength: 490 nm; emission wavelength: 610 nm). Fluorescence signals were quantified using NIS-Elements 3.2 software (Nikon Izasa S.A, L'Hospitalet de Llobregat, Spain).

Statistical analysis

Values are expressed as mean \pm S.E.M. A one-way analysis of variance was used to determine the difference between groups. When an interaction effect was found, multiple comparisons were made using the Tukey method, and 'post hoc' test was performed. The significance has been considered at * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, as indicated in each case. GraphPad Prism v5.0 (GraphPad Software, San Diego, CA, USA) was used for statistical analysis and graphic representations.

Results

Effect of extracellular histones on the endothelial production of NO, PGI2 and TXA2

The first objective of this work was to investigate the effect of extracellular histones on the endothelial production of vasoactive compounds, in particular the two main vascular prostanoids, PGI2 and TXA2, and NO. HUVECs were exposed for 4 hrs to increasing concentration of extracellular histones (1, 10 and 100 ng/ml and 1, 10, 25, 50 and 100 $\mu\text{g/ml}$).

No effect was observed in PGI2 production at low concentrations of histones from 1 ng/ml to 25 $\mu\text{g/ml}$. However, the production of PGI2 increased in a dose-dependent manner at 50 and 100 $\mu\text{g/ml}$ ($P < 0.05$). This increment was up to $62 \pm 8\%$ in cells exposed to 50 $\mu\text{g/ml}$ and up to $420 \pm 97\%$ in cells exposed to 100 $\mu\text{g/ml}$, compared to non-treated cells (Fig. 1A). In contrast, the production of TXA2 by histone-treated HUVEC decreased only at 100 $\mu\text{g/ml}$ ($P < 0.001$) without changing at any other concentration assayed (Fig. 1B).

Using the same conditions described above, increasing concentrations of extracellular histones resulted in a significant decrease of NO production only at 50 ($22 \pm 3\%$, $P < 0.05$) and 100 $\mu\text{g/ml}$ ($26 \pm 2\%$, $P < 0.01$) histones without changes after treatment from 1 ng/ml to 25 $\mu\text{g/ml}$ (Fig. 1C).

Effect of extracellular histones on gene and protein expression of prostanoid pathway

mRNA and protein expression levels of the enzymes involved in PGI2 and TXA2 production were determined. Histone-exposed

HUVEC decreased COX-1 mRNA in a dose-dependent manner (Fig. 2A). At low concentrations of histones, from 10 to 25 $\mu\text{g/ml}$, mRNA COX-1 did not change but the expression decreased up to $24 \pm 7\%$ when cells were exposed to 50 $\mu\text{g/ml}$ ($P < 0.05$) and $29 \pm 7\%$ when cells were exposed to 100 $\mu\text{g/ml}$ of histones ($P < 0.05$). However, COX-2 mRNA expression increased up to $118 \pm 19\%$ at 50 $\mu\text{g/ml}$ ($P < 0.05$) and $379 \pm 66\%$ at 100 $\mu\text{g/ml}$ of histones ($P < 0.001$).

In the prostanoid pathway, the COX products cyclo-endoperoxides PGG2 and PGH2 are rapidly converted in the active compounds PGI2 and TXA2 by means of the specific synthases PGIS and TXAS, respectively. Results from quantitative qRT-PCR analysis showed differences in PGIS mRNA expression, while TXAS mRNA expression remained unaltered (Fig. 2A). Histone-treated HUVEC increased PGIS mRNA levels up to $77 \pm 5\%$ at 50 $\mu\text{g/ml}$ ($P < 0.001$) and up to $96 \pm 6\%$ at 100 $\mu\text{g/ml}$ histones ($P < 0.001$). These data were in accordance with changes observed in PGI2.

Moreover, mRNA expression profile was supported with protein expression analysis determined by Western blot (Fig. 2B). HUVEC exposed to histones shown unaltered COX-1 protein expression (although a tendency to decrease with higher histone concentrations exists), while COX-2 was significantly increased when endothelial cells were exposed at 50 and 100 $\mu\text{g/ml}$ histones. COX-2 protein expression increased up to $43 \pm 5\%$ at 50 $\mu\text{g/ml}$ histones ($P < 0.05$) and up to $94 \pm 56\%$ at 100 $\mu\text{g/ml}$ histones ($P < 0.01$) above control values (Fig. 2B). Regarding specific synthases, mRNA PGIS levels were accompanied by an increment in the amount of PGIS protein expression, also at 50 $\mu\text{g/ml}$ ($36 \pm 4\%$, $P < 0.05$) and 100 $\mu\text{g/ml}$ histones ($51 \pm 6\%$, $P < 0.01$, Fig. 2B). Finally, no changes in TXAS protein expression were found (Fig. 2B).

Therefore, these results demonstrated that HUVECs exposed to extracellular histones show a modulation in COX pathway, mainly by an up-regulation of COX-2 and PGIS that, in turn, could cause an increase in PGI2 production. In fact, PGI2 production in 50 $\mu\text{g/ml}$ histone-treated endothelial cells in the presence of the selective COX-2 inhibitor celecoxib (10 $\mu\text{mol/l}$) completely reversed the effect triggered by extracellular histones (Fig. 2C) supporting the involvement of COX-2 in the observed effect.

Effect of extracellular histones on gene and protein expression of NO pathway

As described above, extracellular histones decreased NO production in HUVEC (Fig. 1C). To evaluate the synthetic pathway of NO, eNOS mRNA and protein expression were determined in HUVEC exposed to increasing concentrations of histone during 4 hrs. eNOS mRNA levels shown a dose-dependent decrease at 50 and 100 $\mu\text{g/ml}$ of histones, $32 \pm 5\%$ ($P < 0.01$) and $38 \pm 5\%$ ($P < 0.05$), respectively (Fig. 3A), result supported by eNOS protein expression (Fig. 3B). Relative levels assessed by densitometry reveal a significant decrease in eNOS protein expression at 50 and 100 $\mu\text{g/ml}$ histones ($P < 0.05$).

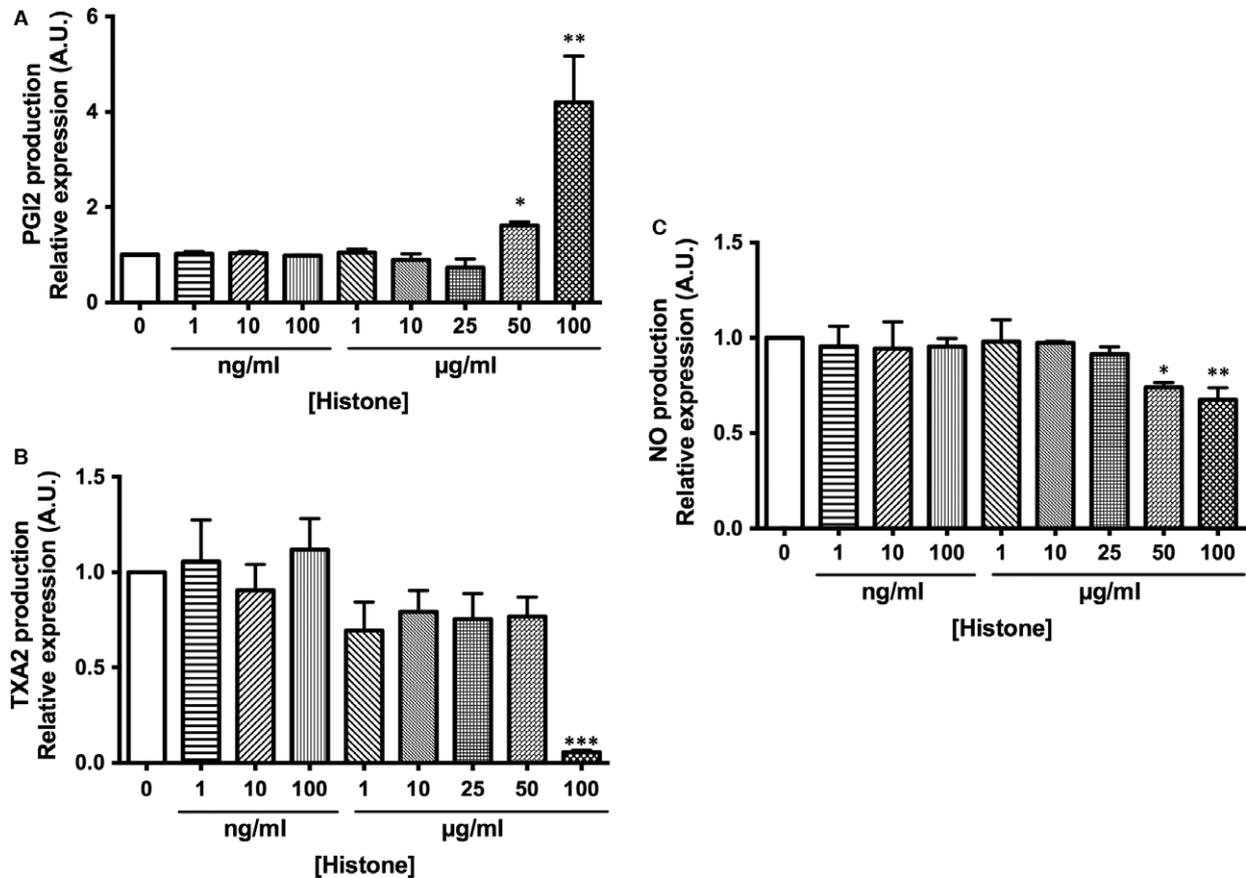


Fig. 1 Extracellular histone-treated HUVEC alter PG12 and TXA2 release and decrease NO production. **(A and B)** HUVECs were exposed to different concentrations of histones for 4 hrs. Cultured medium was then collected, and PG12 and TXA2 concentration was measured by enzyme immunoassay. Data are expressed as mean \pm S.E.M. of $n = 8-10$ from three to five independent experiments. **(C)** HUVEC incubated with different concentrations of histones for 4 hrs were preloaded for 40 min. with the NO probe DAF-FM for NO production determination. Data are expressed as mean \pm S.E.M. of $n = 6-8$ from three to five independent experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ versus histones 0 $\mu\text{g/ml}$.

Role of COX-2 activity in NO production in histone-treated HUVEC

As stated before, histones affect COX and eNOS pathways. Previous reports described an interaction between both pathways [17]. To investigate whether COX activity is related to NO pathway down-regulation, histone-treated HUVECs were incubated with the COX-2 selective inhibitor celecoxib (10 $\mu\text{mol/l}$) and eNOS expression was determined. Results indicated that COX-2 inhibition significantly reversed the reduction in eNOS protein levels induced by 50 $\mu\text{g/ml}$ histones ($P < 0.05$, Fig. 4A).

Moreover, COX have also been described as superoxide (O_2^-)-generating enzymes [18]. Thus, we investigated whether COX-2 activity induced O_2^- production in histone-treated HUVEC. Extracellular histones (50 $\mu\text{g/ml}$) induced O_2^- production ($73 \pm 11\%$, $P < 0.001$) that was reversed by the inhibitor of COX-2, celecoxib (10 $\mu\text{mol/l}$, Fig. 4B), suggesting an involvement of COX-2 in the O_2^- production

induced by histones. To further check whether O_2^- was affecting eNOS expression, incubation with the superoxide dismutase mimetic agent tempol (100 $\mu\text{mol/l}$) reversed eNOS protein levels in 50 $\mu\text{g/ml}$ histone-treated endothelial cells ($P < 0.05$, Fig. 4C).

Finally, the involvement of COX-2-dependent O_2^- production in NO release by histone-treated HUVEC was evaluated. The effect of COX-2 inhibition counteracted the reduced levels of NO production induced by 50 $\mu\text{g/ml}$ histones ($P < 0.05$, Fig. 4D). Similar effect was observed after treatment with tempol (100 $\mu\text{mol/l}$), which also abrogated NO decreased levels induced by 50 $\mu\text{g/ml}$ extracellular histones ($P < 0.05$, Fig. 4D). These results reinforced the role of increased O_2^- produced by COX-2 in decreased NO levels.

Discussion

In the present study, we demonstrated that extracellular histones are implicated in the release of vasoactive mediators in human

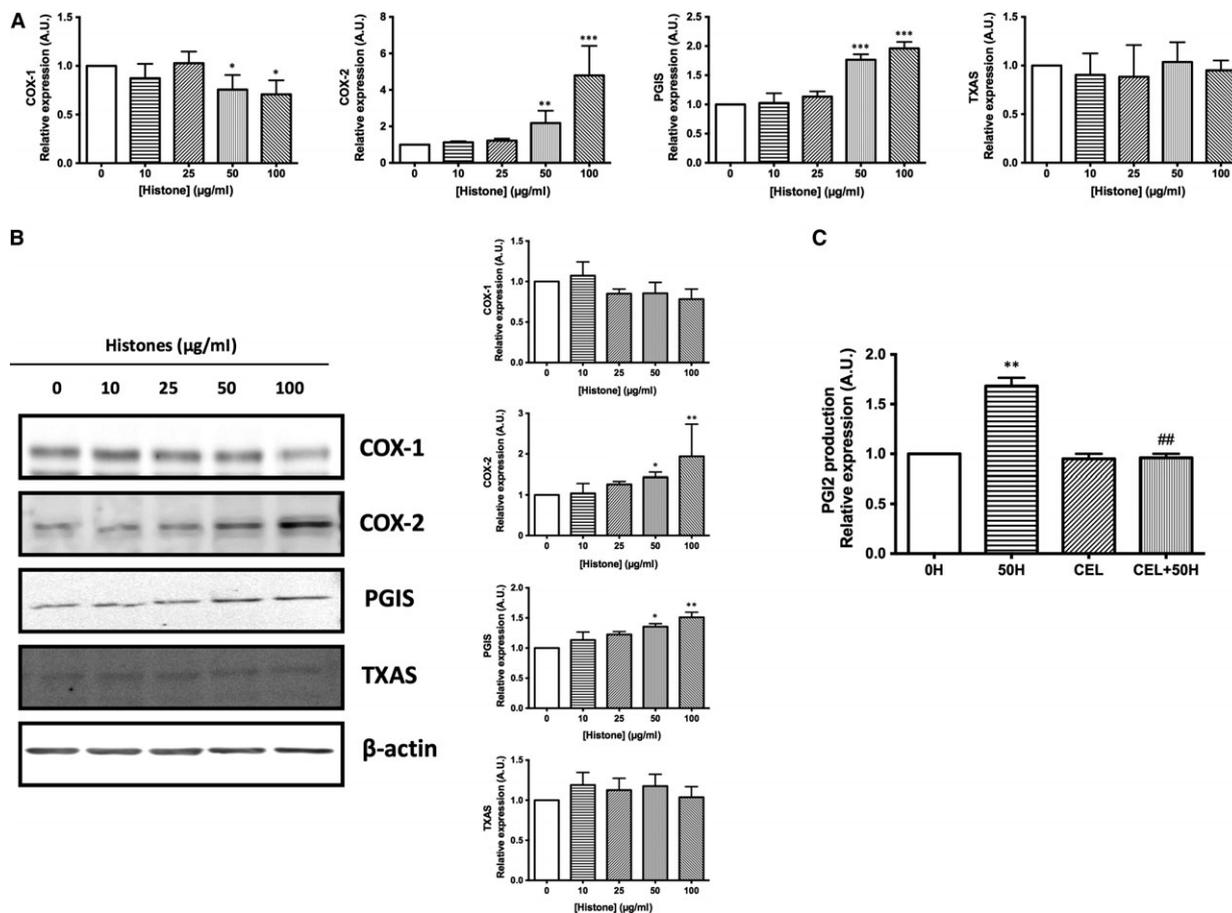


Fig. 2 Extracellular histones alter HUVEC prostanoid production through up-regulation of COX2-PGI2 pathway. **(A)** HUVEC were exposed to 10–100 $\mu\text{g/ml}$ of histones for 4 hrs. Relative COX-1, COX-2, PGIS, TXAS expression was determined by qRT-PCR. Data are expressed as mean \pm S.E.M. of $n = 8$ –10 from three to five independent experiments. **(B)** Protein extracts (20 μg protein) from cultured HUVEC incubated at different concentrations of histones for 4 hrs were loaded on SDS-PAGE gels and analysed by Western blotting using anti-COX-1, anti-COX-2, anti-PGIS and anti-TXAS. β -actin was used as loading control. One representative experiment of three performed is shown. Relative levels assessed by densitometry are presented. **(C)** HUVECs were exposed to 50 $\mu\text{g/ml}$ of histones (50H) for 4 hrs after pre-incubation with celecoxib (CEL), a specific COX-2 inhibitor. Cultured medium was then collected, and PGI2 concentration was measured by enzyme immunoassay. Data are expressed as mean \pm S.E.M. of $n = 3$ –4 from three independent experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ versus histones 0 $\mu\text{g/ml}$ and ## $P < 0.01$ versus histones 50 $\mu\text{g/ml}$.

vascular endothelial cells. First, histone-treated HUVEC showed an increment in PGI2 production in a dose-dependent manner. For TXA2 production, the decrease was only significant at 100 $\mu\text{g/ml}$. Second, extracellular histones increased COX-2 and PGIS mRNA and protein expression, decreased COX-1 mRNA levels but not protein expression and did not change TXAS expression. These results suggest an up-regulation in COX-2-PGIS-PGI2 pathway in those HUVEC exposed to extracellular histones. Third, extracellular histones decreased eNOS expression and NO production in HUVEC. Fourth, we identified COX-2 as an O_2^- -generating enzyme when HUVECs were exposed to extracellular histones and provide new insights on the mechanism by which COX-2 activity may interact with NO production through O_2^- generation. Altogether,

our results suggest a key role of extracellular histones in the modulation of endothelial-dependent factors, such as prostanoid and NO production, and may explain endothelial cell dysfunction observed in histone-mediated pathologies.

Extracellular histones have been implicated in organ injury after trauma [2], autoimmune diseases [19], ischaemic heart disease [20] or sepsis [5]. As a consequence of the endothelium location, the response triggered by endothelial cells to the circulating histone binding is a crucial event in the development of the histone-induced injuries. In this regard, it has been recently described that injuries at lungs and liver induced after extracellular histones challenge are primarily mediated through endothelial damage [21] and induced barrier dysfunction [22]. Increased calcium influx [23] and up-regulation of adhesion

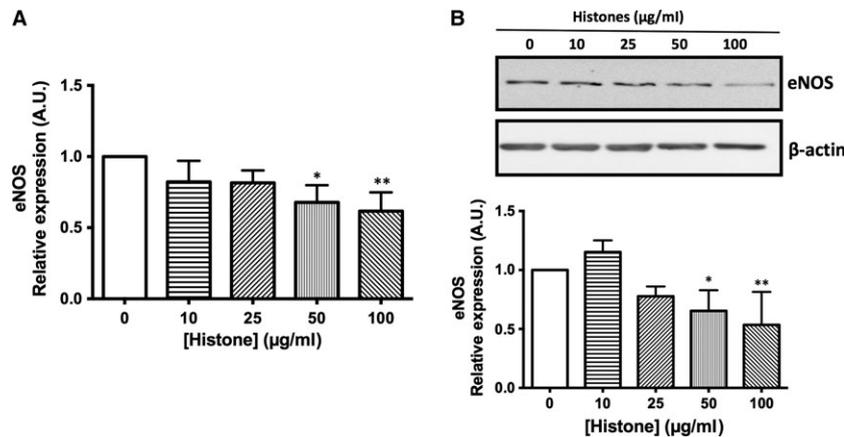


Fig. 3 Extracellular histone-treated HUVECs show decreased eNOS expression. **(A)** HUVEC exposed to increasing concentrations of histones (10–100 µg/ml) for 4 hrs. Relative eNOS expression was determined by qRT-PCR. Data are expressed as mean \pm S.E.M. of $n = 5$ –7 from three to five independent experiments. **(B)** Protein extracts (20 µg protein) from cultured HUVEC incubated at different concentrations of histones for 4 hrs were loaded on SDS-PAGE gels and analysed by Western blotting using anti-eNOS. β -actin was used as loading control. One representative experiment of three performed is shown. Relative levels assessed by densitometry are presented. * $P < 0.05$ and ** $P < 0.01$ versus 0 µg/ml.

molecules [24] have also been observed in histone-treated endothelial cells.

Extracellular histone concentration has been measured in different experimental models. For example, 200 µg/ml of circulating histones was detected in an acute lung injury mice model [25] or 15 µg/ml of H3 in an *E.coli*-induced sepsis model in baboons [5]. Moreover, similar range of circulating histones concentrations has been reported in patients with blunt traumatic lung injury after 4 hrs (10 to 230 µg/ml) [2] and has been detected in plasma of human patients with sepsis (70 µg/ml) [5]. Accordingly, we used a concentration range of extracellular histones (10–100 µg/ml) that showed dose-dependent changes on the endothelial response studied from 50 µg/ml. Nevertheless, we should note that there exists a discordance in the values of circulating histones levels and those used in experimental models, as other authors have reported levels ranging from 0.01 ng/ml to 1600 ng/ml for sepsis and severe sepsis [26, 27]. The wide range of circulating histones in patients suffering different inflammatory diseases described in previous works strongly suggest the need to determine the exact amount of toxic histones. In addition, as stated by Semeraro *et al.* [28], plasma concentrations may underestimate the local amount of histones found at specific sites of cellular release, where they could be much higher.

Our results demonstrate an up-regulation of the COX-2-PGIS pathway and increased synthesis of PGI₂ in response to increasing concentrations of extracellular histones. Prostanoids are essential endothelial mediators for maintaining the vascular homeostasis [29]. Among them, PGI₂ and TXA₂ mediate opposite roles in vascular tone and platelet aggregation. Importantly, PGI₂ is the main prostanoid synthesized by vascular endothelium, playing a crucial role as regulator of correct vascular function [30]. However, PGI₂ overregulation can produce vasodilation and shock [31]. PGIS is constitutively expressed in endothelial cells where it couples with COX-1, although COX-2-dependent PGI₂ production by endothelial cells has been

reported to be modulated *in vitro* by inflammatory cytokines [10]. PGI₂ has also been considered as an endothelial mediator having cytoprotective properties [32]. Different studies have reported that PGI₂ action serves to protect endothelial cells from apoptosis both *in vitro* and *in vivo* conditions [33, 34]. As extracellular histones triggered endothelial cell death using the same concentration range used in our study [23], the increment in PGI₂ release observed could be a compensatory action.

Regarding NO pathway, extracellular histones significantly decrease at 50 and 100 µg/ml the production of NO by HUVEC. We found that NO reduction in histone-treated HUVEC is due, at least in part, to the decrease in eNOS gene and protein expression. In addition to its vasodilatory effect, NO has anti-inflammatory function and protects against vascular injury and leucocyte adhesion to the endothelium [35, 36]. In this regard, decreased NO production levels observed after extracellular histones exposition were in agreement with histone-dependent actions observed in endothelial cells, such as an increased cell adhesion molecules expression on the cell membrane [24] and neutrophil recruitment [37].

Since Salvemini *et al.* [38] described an interaction of NO and COX enzymes in 1993, several studies support a potential ‘crosstalk’ between both prostanoid and NO pathways [39, 40]. COX activity regulation by NO has been described with conflicting results [41, 42] either as positive or as negative regulation of COX activity, and it seems to depend on basal levels of NO released and the cell type used in each study [43]. On the other hand, prostanoid biosynthesis has been also found to modulate NO production, as ibuprofen, a well-known COX inhibitor, increased NO production in arterial endothelial cells [44]. In addition, endothelial cell dysfunction or other NO-depleting situations have been related to a compensatory PGI₂ production [45]. The interaction between both pathways has also been related to cellular redox status as reactive oxygen species, such as O₂⁻, can regulate NO bioavailability [46].

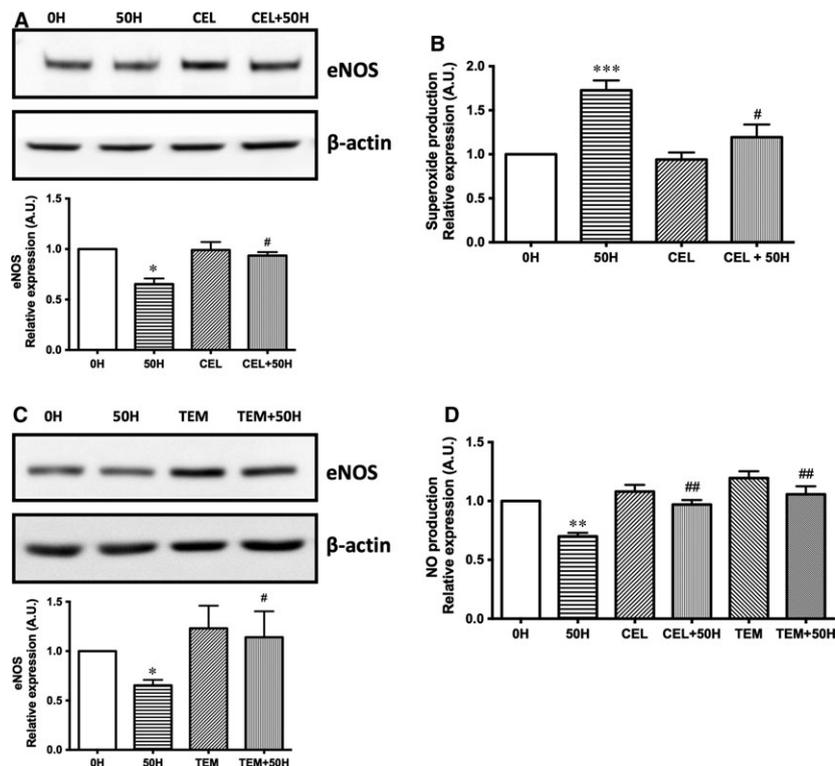


Fig. 4 COX-2, through anion superoxide production, is involved in NO production decrease in extracellular histone-treated HUVEC. **(A)** HUVECs were exposed to 50 $\mu\text{g/ml}$ of histones (50H) for 4 hrs after 1 hr incubation with celecoxib (CEL), and protein extracts (20 μg protein) were loaded on SDS-PAGE gels and analysed by Western blotting using anti-eNOS. β -actin was used as loading control. One representative experiment of five performed is shown. Relative levels assessed by densitometry are presented. **(B)** Intracellular superoxide levels were determined by DHE oxidation as described in Materials and Methods. Results (mean \pm S.E.M. of $n = 4-5$ from three to five independent experiments) **(C)** Histone (50 $\mu\text{g/ml}$)-treated HUVECs (50H) were incubated with tempol (TEM), and protein extracts (20 μg protein) were loaded on SDS-PAGE gels and analysed by Western blotting using anti-eNOS. β -actin was used as loading control. One representative experiment of five performed is shown. Relative levels assessed by densitometry are presented. **(D)** HUVEC incubated with celecoxib (CEL) and tempol (TEM) and treated with 50 $\mu\text{g/ml}$ of histones (50H) for 4 hrs were preloaded for 40 min. with the NO probe DAF-FM to NO production determination. Data are expressed as mean \pm S.E.M. of $n = 5-6$ from three to four independent experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ versus histones 0 $\mu\text{g/ml}$ and # $P < 0.05$; ## $P < 0.01$ versus histones 50 $\mu\text{g/ml}$.

O_2^- are released during COX activity as a consequence of their ability to co-oxidize substances such as NADPH [18]. In this regard, COX-2 has been demonstrated to be an important source of vascular O_2^- production under inflammatory conditions [47–49]. In our study, histone-treated HUVEC showed an increment in COX-2 expression, along with an enzymatic activity, in accordance with the increased PG12 levels observed. Moreover, using celecoxib as a specific inhibitor of COX-2, we demonstrate that COX-2 is involved in O_2^- production increment induced when HUVECs are exposed to extracellular histones, consistent with earlier studies using superoxide dismutase in hepatic endothelial cells under inflammatory conditions [50].

Our findings demonstrate that incubation of histone-treated endothelial cells with celecoxib restored eNOS expression levels. These results are in accordance with those obtained by Fleener *et al.* [51] where O_2^- depletion restored eNOS expression. In addition, histone-treated endothelial cells exposed to tempol, a superoxide dismutase mimetic, also restored eNOS expression

levels, suggesting a role of COX-2-dependent O_2^- in the results observed. We have also shown that inhibition of COX-2 with celecoxib and decreases of O_2^- by tempol restore NO levels induced by extracellular histones. Similar results were obtained in indomethacin-treated ageing aortas where NO bioavailability was restored after COX inhibition [17].

In conclusion, our findings provided evidence that extracellular histones induce concentration-dependent changes in the two main vasoactive mediators, resulting in a decrease in NO levels and a shift in prostanoid release (Fig. 5). Histone-treated endothelial cells show higher PG12/TXA2 ratio through an increment of PG12 production *via* up-regulation of COX-2-PGIS pathway. Moreover, the increase in intracellular superoxide levels observed in histone-treated HUVEC, at least in part produced by COX-2 activity, contributes to a decreased NO bioavailability. Therefore, the molecular mechanisms described in this work could provide new insight on vascular modulation in pathologies in which extracellular histones are involved.

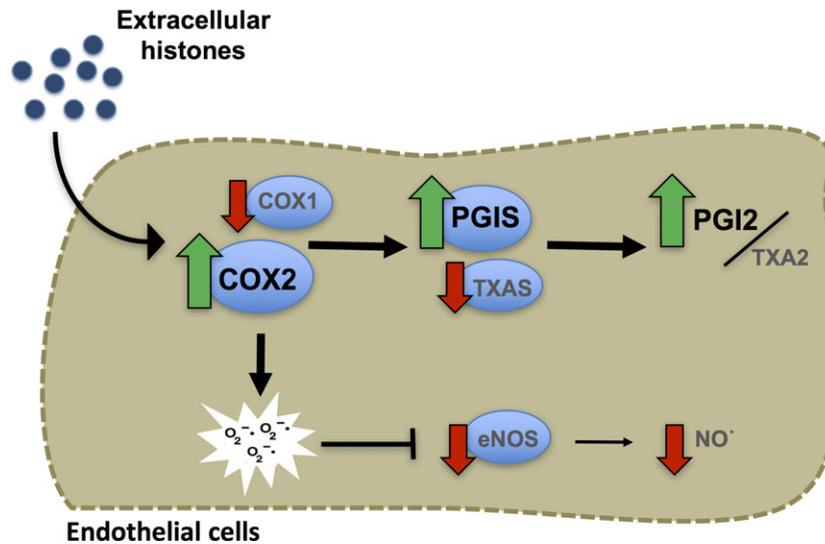


Fig. 5 Extracellular histones modulate prostanoid and NO pathways in human endothelial cells. Histone-treated endothelial cells show higher PGI₂/TXA₂ ratio through an increment of PGI₂ production *via* up-regulation of COX-2-PGIS axis. Extracellular histones increase superoxide levels, due to COX-2 activity, and contribute to a decreased NO bioavailability.

Acknowledgements

This work was supported by Spanish Ministerio de Economía y Competitividad, Instituto de Salud Carlos III and FEDER-ERDF (grants FIS PI10/00518, PI13/00617, and RD12/0042/0052), the Center for Biomedical Network Research (CIBER of Rare Diseases; INGENIO2010), INCLIVA Biomedical Research Institute and Generalitat Valenciana (grant number GV/2014/132) and the Grand Challenges Canada. D.P.-C. and J.S.I.-C. are recipients of 'Atracció de Talent' fellowship from University of Valencia. D.P.-C.,

C.B.-B. and J.S.I.-C. performed the research; D.P.-C., C.H. and S.N. analysed the data; D.P.-C., F.V.P., J.L.G.-G., C.H. and S.N. designed the research study and wrote the manuscript. All authors have read and approved the final version of the article.

Conflict of interest statement

The authors confirm that there are no conflict of interests.

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