Antiinflammatory and antioxidant effects of H_2O_2 generated by natural sources in Il1β-treated human endothelial cells

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ABSTRACT

Specific reactive oxygen species (ROS) from different sources, might lead to different and even opposite, cellular effects. We studied the production of specific ROS resulting from the exposure of human umbilical veins endothelial cells (HUVEC) to H_2O_2 derived from the natural antioxidant epigallocathechin gallate (EGCG) or from the exposure to IL-1β using a fluorogenic probe and flow cytometry, and evaluated by western blot analysis and immunocytochemistry the associated expression of transcription factors sensitive to both inflammatory and oxidative stress, such as NF-kB and Nrf2, and some downstream activated genes such as cyclooxygenase-2 (COX-2) and hemeoxygenase 1 (HO-1). The results obtained showed that exogenously–generated H_2O_2 induce anti-inflammatory and antioxidant effects in HUVECs counteracting the pro-inflammatory and pro-oxidant effect of IL-1β related to the production of superoxide anions. The underlying mechanisms resulting from the extracellular production of H_2O_2, include 1) Nrf2 nuclear translocation and the enhanced expression of antioxidant enzymes such as HO-1, and 2) the previously unreported inhibition of NF-kB and COX-2 expression. Overall, these findings provide evidence that the production of specific reactive oxygen species finely tunes endothelial cell function and might be relevant for the reappraisal of the effects of exogenous antioxidants in the context of cardiovascular diseases).

Keywords

Endothelial cells, reactive oxygen species, oxidative stress, Interleukin-1β, NFkB, antioxidants

INTRODUCTION

We previously demonstrated that short term challenge with extracellular generated H_2O_2 resulting from epigallocatechin-3-gallate (EGCG) increases COX-1 but not COX-2 prostacyclin (PGI_2) production by endothelial cells [1].

The present study aimed at evaluating the effects of EGCG–generated H_2O_2 in human endothelial cells in the presence of a proinflammatory stimulus. In particular, we looked at the production of specific reactive oxygen species (ROS) and at the expression of transcription factors sensitive to both inflammatory and oxidative stress, such as NF-kB and Nrf2, and some downstream activated genes such as COX-2 and HO-1. A simultaneous exposure to pro-inflammatory mediators and ROS is likely to occur in inflammatory states and for instance, it is well accepted that endothelial damage characterized by the acquisition of a pro-inflammatory phenotype worsened by oxidative stress is a hallmark in the development and progression of cardiovascular disease [2]. Indeed, ROS induce the expression of several adhesion molecules, which in turn promote the adhesion of inflammatory cells [3].

Within a specific cell type, ROS may derive by a number of different sources, either from biological processes that release ROS as a byproduct (e.g. oxidative phosphorylation in mitochondria), or from processes that generate ROS intentionally (either in molecular synthesis or breakdown, as part of a signal transduction pathway, or as part of a cell defense mechanism). Among them, ROS and in particular superoxide anion (O_2^-) may be also produced by NAD(P)H oxidases, sometimes in response to inflammatory cytokines such as IL-1β [4,5]. In addition, ROS may also be generated by antioxidant molecules as a first step of an antioxidant signal [6]. Depending on the level of ROS and possibly also on the specific ROS produced [7], different redox-sensitive transcription factors are activated and coordinate distinct, cell specific biological responses. On the one side, a low and

quickly reversible/transient oxidative stress generated by antioxidant polyphenols such EGCG [8], induces nuclear factor erythroid 2-related factor 2 (Nrf2), a transcription factor implicated in the transactivation of genes coding for antioxidant enzymes [9,10]; on the other side, an intermediate amount of ROS can trigger an inflammatory response through the activation of NF-kB [7]. In addition, proinflammatory cytokines such as IL-1β and TNFα have been reported to produce ROS and in turn to activate NF-kB and downstream inflammatory genes such as COX-2, in several cellular models [11]. The redox dependence of NF-kB activation by IL-1β was first shown by Bonizzi and colleagues, which demonstrated how ROS production was required for NF-kB activation by IL-1β in both lymphoid and monocytic cells, but not in epithelial cells [12].

ROS affect NF-kB signaling in several ways; for instance ROS often stimulate the NF-κB pathway in the cytoplasm, but inhibit NF-κB activity in the nucleus [4,13]. Moreover, ROS from different origins have been reported to both activate or to repress NF-κB signaling and there are apparently contradictory information on whether H_2O_2 activates or inhibits NF-κB [7,14]. Similarly, there are few and inconsistent data on EGCG and NFkB activation in endothelial cells [15,16].

The toxic buildup of ROS and cellular oxidation is usually alleviated by enzymes such as the superoxide dismutases (SOD) and catalase, which manage the excessive production of O_2^- and H_2O_2, respectively [17]. In particular, it has also been suggested that cells respond to oxidative stress by activating Nrf 2-antioxidant response element (ARE)-mediated defensive cellular response. The ARE-regulated genes are involved in the production of direct antioxidant (e.g. HO-1) as well as in the direct inactivation of ROS (e.g. catalase, MnSOD). There are a number of compounds derived from natural sources that act as ARE inducers [6] and EGCG has also been recently described to activate Nrf2 and downstream ARE-containing genes in specific cells [18,19].

Thus, there may be a difference in the contribution of ROS to various cellular processes, which in turn could limit the effectiveness of specific antioxidants. Moreover, not ROS *per se*, but rather an unopposed generation of specific ROS may be detrimental for the endothelial integrity, while others ROS (e.g. H_2O_2) could determine beneficial effects, especially under specific circumstances such as inflammation. Therefore, a better understanding of how the type and the temporal aspects of ROS production could both affect the inflammatory response and tune the synthesis of antioxidant enzymes and/or oxidative stress in endothelial cells under inflammatory conditions, is of potential relevance to clarify the final effects of oxidative stress in the setting of cardiovascular disease.

MATERIALS AND METHODS

Cell culture

Human umbilical vein endothelial cells (HUVECs) obtained as previously published [20], were grown in medium 199 (M199, Invitrogen, S. Giuliano Milanese, Milan, Italy) supplemented with 15% FCS (Euroclone; Pero, Milan, Italy), gentamicin (40 g/ml, Invitrogen), endothelial cell growth factor (25 μg/ml), and heparin (100 μg/ml), at 37°C in a humidified 5% CO_2 atmosphere. Cells were identified as endothelial by their morphology and the presence of CD31-related antigen. All experiments were performed on cells at the second passage. HUVECs were seeded at equal density either in 24-well plates (7×10^4/well) or in 6-well plates (3×10^5/well) and incubated in M199 supplemented with 5% FCS. Selected experiments were carried out in the presence of EGCG (1–100 μM, Sigma), 2-tert-butyl-4-quinone (BQT, Sigma), catalase (CAT; 300 U/mL, Sigma), diphenyleneiodonium chloride (DPI, 1-10 μM, Sigma), IL-1β (0.2-2ng/mL, Roche). Inhibitors were added 30 min before the stimulus.

Measurement of reactive oxygen species (ROS)

ROS levels were determined by flow-cytometry (Beckman Coulter Epics XL Flow Cytometer) using 2′,7′-dichlorfluorescein-diacetate (DCFH-DA, Sigma-Aldrich), a cell membrane-permeable fluorogenic probe. The acetate groups of this probe are enzymatically cleaved inside living cells. The probe can then be oxidized by intracellular oxidants (ROS) to give a product, DCF, which emits a strong, green fluorescence (λ_{ex} = 490nm; λ_{em} = 529 nm). The fluorescence intensity increases in proportion to the level of cellular oxidants and is expressed as percent increase *versus* basal values. ROS were evaluated in cells treated with either EGCG (10–100 μM) or Il-1β (2 ng/mL) for different periods (15 min-24 hours). For short term experiments (less than 1 hour), cells were incubated for 30 minutes with DCFH-DA before the further challenge with the specific stimulus. For long term experiments (more than 1 hour) DCFH-DA loading was performed 30 minutes before the end of the incubation time. To detect the potential ability of H_2O_2/EGCG to inhibit the production of ROS in the presence of Il-1β, the cells were pretreated with EGCG for 30 min. Subsequently, Il-1β (2 ng/mL) was added, and the cells were incubated for a further 6-24 h. Selected experiments were performed with catalase (CAT; 300 U/ml, Sigma) or diphenyleneiodonium chloride (DPI, 1-10 μM, Sigma) added 30 min before the stimulus.

Western blot analysis

At the end of incubations, cells were harvested in lysis buffer [50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1% (v/v) Nonidet P-40; 25 mM NaF; 0.5% (w/v) sodium deoxycholate; 10% (w/v) SDS; 1 mM EGTA; 1 mM phenylmethylsulfonyl fluoride; 1 mM orthovanadate; and 10 mM sodium pyrophosphate, supplemented with Complete protease inhibitor mixture obtained from Roche, Mannheim, Germany]. After quantization by Lowry's method, equal amounts of cell protein (20-40 μg) were loaded onto 10% SDS-acrylamide gels. At the end of the run, proteins were transferred to

polyvinylidene difluoride (PVDF) membranes and incubated with polyclonal antibodies against MnSOD (1:1000, Cell Signaling), HO-1 (1:1000, Cell Signaling), IKBα (1:1000, Cell Signaling) pIKBa (1:1000, Cell Signaling) and COX-2 (1:400, Cayman Chemical) overnight and then with suitable peroxidase-conjugated secondary antibodies for 1 h. Proteins were detected by chemiluminescence (Amersham Biosciences, Piscataway, NJ, USA). Loading control was performed using actin immunodetection. In selected experiments cytoplasmic and nuclear proteins were extracted from HUVECs using a Nuclear protein extraction KIT according to the manufacturer's instructions (ProteoJet, Fermentas) and Nrf2 was measured by Western blot analysis (1:500, Abcam).

NF-kB nuclear translocation

HUVECs were seeded in 24-well plates on a sterile cover slip previously placed on the well bottom. Cells were treated with Il-1β 2ng/mL for 45 min, the culture medium was removed and cells were washed twice with sterile PBS to remove any residue of the medium. Cells were then fixed with 4% paraformaldehyde for 10 min at room temperature, and washed three times with sterile PBS to remove the excess of paraformaldehyde. In order to saturate aspecific binding sites, cells were blocked for 45 min with a permeabilization solution of PBS supplemented with 1% FBS and 0.1% Triton and further treated for 30 min with a solution of H_2O_2 (0.3%). Subsequently, the solution was removed and without washing, the p65 primary antibody was added in each well (1:50 in PBS containing 2% FBS, Cell Signaling) and cells were incubated for 1 h at room temperature. At the end of the incubation, cells were washed three times with a PBS solution and incubated with a biotinylated secondary antibody (1:1000, Vector) for 30 min. After shortly washing with PBS solution, cells were incubated for further 30 min with streptavidine peroxidase (1:1000, Vector). After washing with PBS solution, positive cells were detected with 3,3'-diaminobenzidine detection system using Vector VIP, according to manufacturer’s instructions (Vector VIP, Vector).

The cover slips were removed and turned over Mowiol and at last stored at 4°C away from light until image acquisition using a Nikon microscope (Nikon Eclipse, 200x magnification).

PGE_2 and PGI_2 assay

After incubation, the culture medium of EGCG-treated cells were collected and centrifuged at 10,000 *g* for 5 min. Prostaglandin E_2 (PGE_2) or 6-keto $PGF_{1\alpha}$, the stable hydrolysis product of PGI_2, was measured with specific EIA kits (Cayman Chemical) according to the manufacturer's instructions.

MTT cell viability assay

HUVECs were seeded in 96-well plates ($1.5x10^4$/well) and incubated for 24 hours in the absence or presence of different concentrations of EGCG. 10 µL of MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide 5 mg/mL in Dulbecco's Phosphate Buffered Saline, pH=7.4) was added to each well 4 hours before the end of treatment at a final concentration of 5mg/ml. Afterwards the incubation medium was removed and the formazan crystals were dissolved in 100 µL of DMSO. Absorbance at 570nm-630 nm was measured in a multilabel plate counter (VICTOR2–Wallac). Results were expressed as percent of the absorbance observed in the absence of EGCG.

Statistical Analysis

All of the experiments were performed in at least three independent replications; the results are presented as mean values, with error bars representing the standard error (SE) of the average value. A one-way ANOVA test was used for statistical analysis, followed by Bonferroni post hoc tests for multiple comparisons. A value of $p < 0.05$ was considered to be statistically significant. GraphPad Prism 5.02 (GraphPad Software Inc., La Jolla, CA, USA) was used to perform the analyses.

RESULTS

EGCG- and Il-1β differently affect ROS production by HUVECs over time

In order to evaluate the time-dependent effects of IL-1β and EGCG on ROS production, HUVECs were treated with 100 μM EGCG (the concentration previously reported by Toniolo et al [1], to generate amount of H_2O_2capable of affecting cell functions) or 2ng/mL IL-1β for different time intervals in the presence of the cell membrane-permeable fluorogenic probe DCFHDA.

We first observed that in the short term (5-30 min), both EGCG and IL-1β, alone or in combination increased ROS generation by HUVECs (Fig.1). Conversely, challenging the cells for 6 hours, EGCG no longer affected ROS production, while IL-1β effect on ROS was greater with respect to that observed at 30 min (324.9%±44.2 and 146.5%±8.6 vs basal, respectively). Moreover, when HUVECs were treated with IL-1β in the presence of EGCG for 6 or 24 hours, a concentration-dependent decrease in ROS production was observed (Fig. 2A and 2B). Altogether, these results suggest that the ROS/H_2O_2 initially generated by EGCG might be responsible for a delayed antioxidant effect. Viability, as assessed by the MTT test, was not reduced by the exposure to EGCG 1-100 μM for up to 24 hours (data not shown).

Effects of EGCG and IL-1β on redox-sensitive transcription factor activation and downstream gene expression

In order to evaluate whether EGCG-derived H_2O_2 activates redox-sensitive transcription factors, which in turn could be responsible for antioxidant and/or antiinflammatory activity, we first looked at the nuclear translocation of Nrf2 in HUVECs treated with either 100 μM EGCG or 2-tert-butyl-4-quinone (TBQ), a well recognized antioxidant used as positive control. As reported in Fig. 3,

challenging the cells for 1 hour with EGCG increased the nuclear fraction of Nrf2, clearly suggesting that EGCG induced the nuclear translocation of Nrf2. By contrast, IL-1β was not able to affect Nrf2 nuclear translocation (data not shown). Accordingly, after 6 hours EGCG but not IL-1β increased the expression of HO-1, an antioxidant enzyme containing ARE in its promoter, which binds Nrf2. This effect, at least at this time point, was specific for heme oxygenase-1 (HO-1) since neither the expression of glutathione peroxidase (GPx) nor that of superoxide dismutase (MnSOD) was modified by EGCG (Fig. 4A, 4B and 4C). By contrast, IL-1β increased MnSOD without affecting neither HO-1 nor GPX expression.

We then analyzed the effects of EGCG and IL-1β on the activation of NF-kB, another widely recognized redox-sensitive transcription factor. In HUVECs treated with IL-1β the pIkB/IkB protein ratio increased in a time-dependent manner, peaking at 30 minutes (Fig. 5A). The pre-treatment of cells with EGCG-generated-H_2O_2 prevented NF-kB activation, as observed by a decreased pIKB/IKB ratio (Fig. 5B). Accordingly, a significant decrease in p65 nuclear translocation was observed in HUVECs challenged for 45 minutes with IL-1β in the presence of EGCG with respect to untreated cells (Fig. 5C). Moreover, after 6 hours, EGCG (10-100 μM) decreased the expression of IL-1β-induced endothelial COX-2 expression (Fig. 6A) as well as the production of both the prostacyclin metabolite 6-keto-$PGF_{1\alpha}$ and PGE_2 (Fig. 6B and 6C), further suggesting a role for H_2O_2 in tuning the IL-1β effects on endothelial cells.

Overall, this data demonstrates that EGCG and IL-1β exert opposite effects on the activation of both Nrf2 and NF-kB as well as on the expression of some downstream target genes possibly due to the generation of different ROS.

IL1β-produced ROS are inhibited by DPI but not by catalase

In order to further explore the oxygen species involved in IL-1β-mediated pro-oxidant and pro-inflammatory effects, HUVECs were treated with IL-1β in the presence of different antioxidant molecules. At variance with what observed with EGCG (see also [1]), catalase did not affect the generation of ROS induced by the treatment of HUVECs with IL-1β (0.2-2ng/mL) for either 6 or 24 hours (Fig. 7A and 7B). Conversely, challenging cells with IL-1β (0.2-2ng/mL) in the presence of the NADPH inhibitor diphenyleneiodonium chloride (DPI, 1-10 μM) for 6 hours, the generation of ROS was reduced in a concentration-dependent manner (Fig. 7C). Overall, these data indicate that the reactive oxygen species produced by IL-1β in HUVECs are dependent on the activation of NADPH oxidase and thus are mainly represented by superoxide anion.

The NADPH inhibitor DPI and H_2O_2 from EGCG inhibit IL1β-stimulated activation of NF-kB in HUVECs

In order to understand the contribution of specific oxygen species in NF-kB activation, we further analyzed the effects of DPI and catalase on IL1β-induced NF-kB activation. Similarly to what observed for EGCG, treatment of cells with DPI prevented IL-1β-induced NF-kB activation evaluated as a decreased pIkB/IkB ratio (Fig. 8A), suggesting that NFkB activation requires IL-1β-induced ROS production. Conversely, catalase was unable to prevent IL-1β-mediated NF-kB activation (Fig. 8B). More interestingly and quite unexpectedly, catalase counteracted EGCG inhibition of IL-1β-mediated NF-kB activation, clearly suggesting that specific oxygen species are crucial for either activation (superoxide anion) or inhibition (H_2O_2) of NF-kB (Fig. 8B).

DISCUSSION

We demonstrated that short-term exposure to H_2O_2, generated from high concentrations of EGCG, leads to antioxidant and anti-inflammatory effects in HUVECs through the activation of Nrf2 and the inhibition of NF-kB and the downstream-activated genes HO-1 and COX-2, respectively. Consistent with the production of different reactive oxygen species, IL-1β induces a time-dependent activation of NF-kB in HUVECs, which is blocked by DPI. Notably, we provide the previously unreported evidence that IL1β-induced COX-2 and NF-kB activation is also inhibited by H_2O_2 produced by EGCG, and this effect is prevented by catalase.

A simultaneous exposure to pro-inflammatory mediators and ROS is likely to occur in inflammatory states and contributes for instance to endothelial cell dysfunction, which is often associated with cardiovascular disease [7,21,22]. The cellular response to these environmental changes depends on a number of factors such as the amount and the type of ROS produced by a number of sources, such as pro-inflammatory cytokines, as well as by the availability of antioxidant defenses, such as cell-specific antioxidant enzymes [7,17]. In this complex scenario, the use of exogenous antioxidants may affect the cellular response sometimes leading to undesired effects [17,23]. The present paper was aimed at evaluating the effects of specific ROS species likely to be present during inflammatory conditions in the endothelial cells environment by looking at the expression of transcription factors such as NF-kB and Nrf2, sensitive to both inflammatory and oxidative stress, and some downstream activated genes.

EGCG is the major bioactive polyphenol in green tea. EGCG has been reported also by our group to undergo auto-oxidation and to generate H_2O_2, which in turn is known to be responsible for the long-term antioxidant activity [1,19]. With respect to ROS production, different laboratories reported a wide range of variable results in terms of extent and rate of ROS production, which could be explained by the use of different cell culture media and/or time of exposure [24][25]. In

the context of our experimental set-up, we would like to stress the fact that EGCG was used at high concentration that did not affect endothelial cell viability as a natural source of H_2O_2 and we are not implying any potential effect described in the manuscript as a mechanism of the anti-inflammatory action of EGCG. Indeed, even if accumulation and different sources of catechins may result in increased plasma concentrations, the single consumption of the extract of 10 g of green tea (similar to 16 ounces of green tea infusion) yielded plasma concentrations of tea catechins of about 1-3 µM, with a half-life of 5 hours [26]. Nevertheless, ROS generation by EGCG, at concentrations similar to those used in our study, have also been reported to contribute to cytoprotective effects [27] as well as endothelium-dependent relaxation [28].

The role of ROS in NF-kB activation has also been the subject of intense studies leading to conflicting results. The production of ROS upon IL-1β, TNF-α and LPS stimulation has emerged as evidence, but their role in NF-kB activation is still controversial [4,7]. Korn et al. reported that, H_2O_2 is capable of inhibiting TNF-induced NF-kB activation in lung epithelial cells by reducing IKKb activity through oxidation of cysteine residues in the IKK complex [29]. By contrast, it has been shown that ROS production was required for NF-kB activation by IL-1β in both lymphoid and monocytic cells, but not in epithelial cells [12]. Moreover, Ludwig et al. previously reported that 100 µM EGCG does not affect the nuclear translocation of p65 in TNFα-stimulated HUVEC [15], whereas more recently Li et al. demonstrated an inhibitory effect of EGCG on NF-kB activation in cerebral endothelial cells but the mechanism has not been studied [16]. We here demonstrated for the first time that a short exposure to EGCG-produced H_2O_2 inhibits IL-1β-induced Nf-kB activation in HUVECs. Indeed, EGCG decreased pIkB/IkB ratio, an index of NF-kB activation [11], as well as p65 nuclear translocation. Notably, catalase prevented the effect of EGCG, clearly suggesting that extracellular H_2O_2 produced by EGCG has an inhibitory effect on IL-1β-mediated NF-kB activation. Literature is abundant with apparently contradictory information on whether

H_2O_2 activates or inhibits NF-kB and it is still a matter of debate which ROS mediates NF-kB activation [7,14]. Interestingly, under our experimental conditions, NF-kB activation by IL-1β was also inhibited by the NADPH oxidase inhibitor DPI, demonstrating that NADPH oxidase-derived ROS, in particular superoxide anion, are required for NF-kB activation by IL-1β in HUVECs and further strengthening the specific and opposite role of different oxygen species in NF-kB activation. Accordingly, DPI inhibited IL-1β-generated ROS. In addition, we demonstrated an increased MnSOD expression in IL-1β-treated HUVEC. It has been already described that the exposure of cells to cytokines such as IL-1β and TNFα leads to the accumulation of toxic reactive oxygen intermediates and, not surprisingly, to the induction of a gene, MnSOD, whose product eliminates these radicals from the cell. In particular, TNFα and IL-1β-mediated induction of MnSOD has been shown to confer protection against myocardial reperfusion injury and tissue damage due to oxidative stress [30]. The mechanisms for the induction of MnSOD expression under all of these conditions is not completely understood. However, activation of the transcription factor NF-kB has been implicated to play a role [31,32].

Overall, as far as NF-kB modulation by specific ROS in HUVECs, weprovide previously unreported data showing that 1) the activation of NF-kB by IL-1β depends on the generation of ROS different from H_2O_2; 2) IL-1β, possibly through NF-kB activation, increases the expression of the antioxidant MnSOD; 3) EGCG-produced H_2O_2 prevents IL-1β-mediated Nf-kB activation.

To further study the effects of exogenously-generated H_2O_2 / H_2O_2 produced from a natural source and IL1-β on endothelial cell activation, we looked at the expression of COX-2, a gene sensitive to both oxidative stress [33–35] and inflammatory stimuli [36], which indeed is also a well known NF-kB target gene in endothelial cells [37]. We demonstrated that, similarly to what observed for NF-kB, IL-1β-enhanced COX-2 expression is inhibited by EGCG. Previously it has been reported that ROS generated by TNFα or exogenously added H_2O_2 to cells for 16 hours activate COX-2 and the

production of 6keto-PGF1α and PGE_2, which in turn were inhibited by the SOD analogue [38]. Interestingly, however, it has been reported that endothelial cells in the presence of IL-1β produce PGI_2 only for short periods and thereafter PGI synthase is inactivated [39]. We here demonstrated that EGCG decreases the production of COX-2 metabolites 6keto-PGF1α and PGE_2 when in the presence of IL-1β. It has also been shown that EGCG inhibited COX-2 expression in linoleic acid-stimulated HUVECs through a mechanism involving ERK1/2 phosphorylation [40]. Indeed, we cannot exclude that mechanisms other than NF-kB are responsible for IL-1β-enhanced COX-2 expression [41]. This is an ubiquitous enzyme up-regulated in response to oxidant stress and inflammatory stimuli, and may play an important role in preserving vascular and metabolic homeostasis. Indeed, we also demonstrated that EGCG causes both Nrf2 nuclear translocation and HO-1 induction. Consistent with our data, there is evidence that EGCG can induce HO-1 via activation of Nrf2 in other endothelial cell models [18,19].

Conclusions

In conclusion, extracellular H_2O_2 generated using high concentrations of EGCG exerts anti-inflammatory and antioxidant effects in HUVECs counteracting the pro-inflammatory and pro-oxidant effect of Il-1β. The mechanisms underlining the effects of extracellular H_2O_2 include 1) Nrf2 nuclear translocation and the enhanced expression of antioxidant enzymes such as HO-1, and the previously unreported 2) inhibition of NF-kB and COX-2 expression. Overall, these findings provide evidence that the production of specific reactive oxygen species finely tunes endothelial cell function and might be relevant for the reappraisal of the effects of exogenous antioxidants in the context of cardiovascular diseases.

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FIGURE LEGENDS

Figure 1. Effect of EGCG and IL-1β on ROS production in HUVECs. HUVEC were incubated (30 min) with the cell membrane-permeable, ROS-sensitive fluorogenic probe DHCF-DA, and upon treatment with either EGCG or IL-1β (5-30 min), fluorescence was determined by flow cytometry. Fluorescence intensity is expressed as % of control incubations.

**P<0.01 *vs* control

Figure 2. Effect of EGCG on ROS production induced by IL-1β in HUVECs. HUVEC were treated with EGCG for 30 min, followed by Il-1β (2 ng/mL; panel A: 6 h, panel B: 24 h). Cells were finally incubated with the cell membrane-permeable, ROS-sensitive fluorogenic probe DHCF-DA (30 min), and fluorescence determined by flow cytometry. Fluorescence intensity is expressed as % of Control incubations.

*P<0.05, **P<0.01, ***P<0.001 *v*s Il-1β

Figure 3. Effect of EGCG on Nrf2 nuclear translocation in HUVECs. HUVEC were treated with 100 µM EGCG or 2-tert-butyl-4-quinone (BQT) for 1 h, and nuclear proteins were extracted using a commercially available nuclear protein extraction KIT. Nrf2 protein was then detected by western blotting in the nuclear and cytoplasmic fractions while GAPDH was monitored as loading control. Panel A: representative western blot. Panel B: semiquantitative analysis of nuclear Nrf2; western blot optical density was evaluated using ImageJ 1.47v (NIH, USA) and Nrf2 data were normalized using the optical density of corresponding GAPDH.

*P<0.05 *v*s control

Figure 4. IL-1β and the expression of Heme oxygenase-1 (HO1), Mn superoxide dismutase (MnSOD) and glutathione peroxidase (GPx) in HUVECs: effect of EGCG. HUVEC were treated with IL-1β and/or EGCG for 6 h, and proteins were detected by western blotting. β-actin was monitored

as loading control. Panel A: representative western blot (left side) and semiquantitative analysis (right side) of the expression of HO1 in the presence or absence of IL-1β and/or EGCG. Panel B: representative western blot (left side) and semiquantitative analysis (right side) of the expression of MnSOD in the presence or absence of IL-1β and/or EGCG. Panel C: representative western blot (left side) and semiquantitative analysis (right side) corresponding β-actin.of the expression of HO1 in the presence or absence of IL-1β and/or EGCG. Western blot optical density was evaluated using NIH ImageJ software and data were normalized using the optical density of

*P<0.05, **P<0.01 *vs* control *;* ### P<0.001 vs IL-1β

Figure 5. IL-1β and the activation of Nf-kB in HUVECs: effect of EGCG. HUVEC were treated with IL-1β in the absence (5-30 min) or presence (30 min) of EGCG. IkBα and its phosphorylated form (p-IkBα) were analysed by western blotting and β-actin was monitored as loading control, while nuclear translocation of p65 was monitored by immunocytochemistry. Panel A, upper part: representative western blots of the effects of IL-1β on IkBα and its phosphorylated form; Panel A, lower part: semiquantitative analysis of the changes in the ratio between p-IkBα and IkBα, both normalized for their corresponding β-actin. Panel B, upper part: representative western blots of the effects of EGCG on the changes induced by IL-1β on IkBα and its phosphorylated form; Panel B, lower part: semiquantitative analysis of the changes in the ratio between p-IkBα and IkBα, both normalized for their corresponding β-actin. Western blot optical density was evaluated using NIH ImageJ software and data were normalized using the optical density of corresponding β-actin.Panel C: immunocytochemistry of changes in subcellular p65 distribution upon treatment with EGCG and /or IL-1β. Images were acquired with a Nikon Eclipse TI-S microscope, 40x magnification).

**P<0.01 *vs* control, $ P<0.05 *vs* IL-1β

Figure 6. IL-1β and the expression of COX-2 in HUVECs: effect of EGCG. HUVEC were treated with IL-1β for 6 h, in the absence or presence of EGCG. COX-2 expression was monitored by western blotting and β-actin was used as loading control. Panel A: representative western blots of the effects of EGCG on the induction of COX-2 by IL-1β. Panel B: semiquantitative analysis of the

effects of EGCG on the induction of COX-2 by IL-1β. Panel C: effect of EGCG on the production of 6ketoPGF1α (left side) and PGE_2 (right side) induced by treatment with IL-1β. Western blot optical density was evaluated using NIH ImageJ software and data were normalized using the optical density of corresponding β-actin. Quantitation of eicosanoids was carried out by commercially available EIA.

*P<0.05 *vs* control; $P<0.05 *vs* IL-1β; **P<0.01 *v*s control (Panel A) or IL-1β (Panel B)

Figure 7. Effect of catalase or DPI on ROS production induced by IL-1β in HUVECs. HUVEC were treated with Il-1β (2 ng/mL; panel A and C: 6 h, panel B: 24 h) in the presence or absence of catalase (Panel A and B) or different concentrations of DPI (Panel C). Cells were finally incubated with the cell membrane-permeable, H_2O_2-sensitive fluorogenic probe DHCF-DA (30 min), and fluorescence determined by flow cytometry. Fluorescence intensity is expressed as % of Control incubations.

*P<0.05 vs control; **P<0.01 vs control; ***P<0.001 vs control; ##P<0.01 *v*s IL-1β

Figure 8. IL-1β and the activation of Nf-kB in HUVECs: effect of EGCG and/or DPI and catalase. HUVEC were treated with IL-1β for 30 min, in the presence or absence of test compounds. IkBα and its phosphorylated form (p-IkBα) were analysed by western. Panel A: semiquantitative analysis of the changes in the ratio between p-IkBα and IkBα induced by IL-1β in the absence or presence of EGCG and/or DPI. Panel B: semiquantitative analysis of the changes in the ratio between p-IkBα and IkBα induced by IL-1β in the absence or presence of EGCG and/or catalase. Western blot optical density was evaluated using NIH ImageJ software and data were normalized using the optical density of corresponding β-actin.

*P<0.05 *vs* control; #P<0.05 *vs* IL-1β; **P<0.01 *vs* control; $$P<0.01 *vs* IL-1β+EGCG