Endothelial Dysfunction in Small Arteries of Essential Hypertensive Patients

Role of Cyclooxygenase-2 in Oxidative Stress Generation

Agostino Virdis, Alessandra Bacca, Rocchina Colucci, Emiliano Duranti, Matteo Fornai, Gabriele Materazzi, Chiara Ippolito, Nunzia Bernardini, Corrado Blandizzi, Giampaolo Bernini, Stefano Taddei

Abstract—Essential hypertensive patients show a reduced nitric oxide availability secondary to oxidative stress generation in peripheral microcirculation. Cyclooxygenase (COX) contributes to reduce nitric oxide availability. We assessed the possible vascular sources of oxidative stress, including COX-1, COX-2, and nicotinamide adenine dinucleotide phosphate oxidase, as determinants of endothelial dysfunction in small arteries isolated from essential hypertensive patients or normotensive controls. Small arteries were dissected after subcutaneous fat biopsies and evaluated on a pressurized micromyograph. Endothelium-dependent vasodilation was assessed by acetylcholine, repeated under NG-nitro-l-arginine methyl ester, SC-560 (COX-1 inhibitor), DuP-697 (COX-2 inhibitor), ascorbic acid, or the nicotinamide adenine dinucleotide phosphate oxidase inhibitors apocynin or diphenylene iodonium. Vascular oxidative stress generation (fluorescent dihydroethidium), COX-1 and COX-2 expression (Western blot), and localization (immunohistochemistry) were also assessed. In controls, response to acetylcholine was blunted by NG-nitro-l-arginine methyl ester (P<0.001) and unmodified by SC-560, DuP-697, or ascorbic acid. In hypertensive patients, relaxation to acetylcholine was blunted, resistant to NG-nitro-l-arginine methyl ester or SC-560, and enhanced (P<0.01) by DuP-697, apocynin, or diphenylene iodonium (P<0.05). Furthermore, in hypertensive patients, response to acetylcholine was normalized by ascorbic acid or apocynin+DuP-697. Intravascular oxidative stress generation was enhanced in hypertensive patients, decreased (P<0.01) by DuP-697, partly attenuated by apocynin or diphenylene iodonium, and prevented by ascorbic acid. Enhanced COX-2 expression and localization in the vascular media of hypertensive patients were also detected. In small resistance arteries of essential hypertensive patients, COX-2 is overexpressed and reduces nitric oxide availability. COX-2 represents a major source of oxidative stress generation, whereas nicotinamide adenine dinucleotide phosphate oxidase plays a minor, but significant, role in promoting superoxide generation. (Hypertension. 2013;62:00-00.) • Online Data Supplement

Key Words: endothelium • microcirculation • nitric oxide • oxidative stress

In healthy conditions, nitric oxide (NO) acts as a modulator of both vascular tone and structure, playing a predominant protective role on the vasculature. In genetic hypertension, a number of endothelium-derived contracting factors have been identified. These include several prostanoids, such as prostaglandins and thromboxane A₂, derived from arachidonic acid by the enzyme cyclooxygenase (COX), and reactive oxygen species (ROS), generated by several enzymatic sources, including nicotinamide adenine dinucleotide phosphate (NAD[P]H) oxidase, xanthine oxidase (XO), uncoupled NO synthase (NOS), or COXs itself.³

Pivotal studies in animal models of genetic hypertension have demonstrated an evident production of COX-dependent endothelium-derived contracting factors accounting for endothelial dysfunction.¹⁴ In humans, essential hypertensive patients (EH) show a reduced endothelial NO availability in several vascular districts, including peripheral microcirculation.³ In this setting, the infusion of nonselective COX inhibitors normalizes the endothelial function⁶⁷ and restores NO availability.⁷ Of note, no additive effects are observed when an antioxidant compound and a COX inhibitor are simultaneously administered in these patients.⁸ These findings demonstrate that the COX pathway actively interferes with NO availability and represents a source of ROS in the peripheral microcirculation of EH. At this time, COX represents the unique pathway identified acting as a source of ROS in

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human hypertension. However, 2 different isoforms of COX are known to exist. In most tissues, COX-1 is constitutively expressed to produce physiological prostanoids, whereas COX-2 is often induced by a number of stimuli, including inflammation or growth factors. Nevertheless, COX-2 is also expressed constitutively in several organs. In particular, within the vasculature, endothelial and vascular smooth muscle cells do express both isoforms, with COX-1 being usually expressed at a higher extent than COX-2. Which of these 2 COX isoenzymes is the predominant isoform contributing to ROS generation in EH remains still undetermined. Therefore, the primary aim of this study was to assess the role of COX-1 and COX-2 as determinants of endothelial dysfunction in small resistance arteries from EH, as well as their cross-talks with NO and ROS. Second, we investigated whether other pathways, including NAD(P)H oxidase, uncoupled NOS, or XO, may participate as adjunctive sources of ROS in small resistance arteries.

Methods

Detailed methods are available in the online-only Data Supplement.

Study Population

The main study included 14 mild-moderate EH and 14 matched normotensive control subjects (NT). An adjunctive group of 7 matched EH was further assessed for control experiments (Table). The protocol was approved by the local ethical committee, and the informed consent was obtained from each participant.

Preparation of Small Arteries and Functional Experiments

All participants underwent a biopsy of subcutaneous fat from the anterior abdominal region, taken during the surgical procedure. Small arteries were isolated and mounted in a pressurized myograph, as previously described. Endothelium-dependent and endothelium-independent relaxations were assessed by cumulative concentrations of acetylcholine (ACh, 0.001–100 μmol/L) and sodium nitroprusside (0.01–100 μmol/L) in vessels precontracted with norepinephrine (1 μmol/L).

Figure 1. Relaxations to acetylcholine±NG-nitro-L-arginine methyl ester (L-NAME), ascorbic acid (Asc. Acid), or both in normotensive control subjects (A) and essential hypertensive patients (B). Data as mean±SEM (n=8 each). *P<0.001.

Figure 2. A. Relaxations to acetylcholine±NG-nitro-L-arginine methyl ester (L-NAME), DuP-697, or both in essential hypertensive patients. B. Relaxations to acetylcholine±L-NAME, apocynin (Apo), or Apo+DuP-697+L-NAME in essential hypertensive patients. Data as mean±SEM (n=8 each). *P<0.001; †P<0.05.
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COX-2 and Endothelial Dysfunction  

To evaluate NO availability and ROS production, curves to ACh were constructed before and after 30-minute incubation with the NOS inhibitor NG-nitro-l-arginine methyl ester (L-NAME; 100 μmol/L; Sigma), the antioxidant ascorbic acid (100 μmol/L; Sigma), or both.

### Influence of COX-1, COX-2 Activity, and Thromboxane-Prostanoid Receptors

Furthermore, in small arteries of EH and NT, the roles of COX-1 and COX-2 on NO availability were assessed by repeating ACh after 30-minute incubation with SC-560 (1 μmol/L, selective COX-1 inhibitor), DuP-697 (1 μmol/L, selective COX-2 inhibitor), and simultaneously L-NAME+SC-560, or DuP-697. In adjunctive vessels of EH (n=7), the roles of COX-1 and COX-2 were assessed by repeating ACh after 30-minute incubation with indomethacin (10 μmol/L, nonselective COX inhibitor; Sigma) or SC-560+DuP-697. The contribution of thromboxane-prostanoid (TP) receptors was tested by repeating ACh after incubation with the TP receptor antagonist SQ-29548 (1 mmol/L; Cayman Chemical).

### Detection of NAD(P)H Oxidase, Uncoupled NOS, and XO

In vessels of EH, ACh was repeated after 30-minute incubation with apocynin (10 μmol/L; Sigma) and diphenylene iodinium (DPI, 100 μmol/L; Sigma), the 2 different NAD(P)H oxidase inhibitors; sepiapterin, a precursor of tetrahydrobiopterin which is a cofactor for NO synthesis (100 μmol/L; Sigma); or allopurinol, the XO inhibitor (100 μmol/L; Sigma).

### Detection of Vascular Superoxide Anion Generation

The in situ production of superoxide anion was measured by means of the fluorescent dye dihydroethidium (Sigma), as previously described. Each segment was analyzed simultaneously after incubation with ascorbic acid (100 μmol/L), DuP-697 (100 μmol/L), SC-560 (100 μmol/L), apocynin (100 μmol/L), DPI (100 μmol/L), allopurinol (100 μmol/L), sepiapterin (100 μmol/L), or Krebs solution.

### Western Blot Analysis of COX-1 and COX-2

Western blot analysis was carried out to detect COX-1 and COX-2 protein expression in small arteries of EH and NT.

### Immunostaining of COX Isoforms

Immunohistochemical analysis of COX-1 and COX-2 in small vessels from EH and NT was detected, as previously described.

### Data Analysis

Results are presented as mean±SEM and analyzed by repeated measures ANOVA, followed by Student–Newman–Keuls test, or by 2-sided unpaired Student t test where appropriate. A value of P<0.05 was considered statistically significant. ACh-induced and sodium nitroprusside–induced responses were expressed as maximal percentage increments of lumen diameter or pEC50 values (Table S1 in the

### Table. Clinical Characteristics and Vascular Lumen Diameters of Study Populations

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Essential Hypertensive Patients (n=14)</th>
<th>Normotensive Subjects (n=14)</th>
<th>Essential Hypertensive Patients (n=7)</th>
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</thead>
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<tr>
<td>Age, y</td>
<td>50.9±7.7</td>
<td>49.6±8.0</td>
<td>50.4±7.2</td>
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<td>Men/women</td>
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<td>5/9</td>
<td>3/4</td>
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<td>Body mass index, kg/m²</td>
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<td>Systolic BP, mmHg</td>
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<td>125.7±5.3*</td>
<td>150.8±7.9</td>
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<tr>
<td>Diastolic BP, mmHg</td>
<td>98.9±3.1</td>
<td>78.3±4.5*</td>
<td>99.5±4.6</td>
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<td>Heart rate, bpm</td>
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<td>Total cholesterol, mg/dL</td>
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<td>214.2±17.6</td>
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<td>HDL cholesterol, mg/dL</td>
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<td>Triglycerides, mg/dL</td>
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<td>119.1±15.9</td>
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<tr>
<td>LDL cholesterol, mg/dL</td>
<td>136.9±16.8</td>
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<td>139.9±18.8</td>
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<td>Plasma glucose, mg/dL</td>
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<td>81.4±7.8</td>
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<tr>
<td>eGFR, mL/min per 1.73 m²</td>
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<td>97.8±10.0</td>
<td>92.5±12.1</td>
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<tr>
<td>Resting lumen diameter, μm</td>
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<td>213±8</td>
<td>194±8</td>
</tr>
<tr>
<td>Lumen diameter precontracted with norepinephrine, μm (%)</td>
<td>68±3 (64)</td>
<td>73±4 (65)</td>
<td>67±5 (65)</td>
</tr>
</tbody>
</table>

BP indicates blood pressure; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; and LDL, low-density lipoprotein. *P<0.001 vs other groups.

**Figure 3.** Percentage inhibitory effect of NG-nitro-l-arginine methyl ester (L-NAME) on maximal relaxant response to acetylcholine (ACh)±ascorbic acid (Asc. acid), DuP-697 (Dup), apocynin (Apo), or Apo+Dup. Data as mean±SEM (n=8 each). †P<0.01 vs other groups; †P<0.05 vs controls or ascorbic acid; ‡P<0.05 vs Dup-697.
online-only Data Supplement), where pEC\textsubscript{50} is −logEC\textsubscript{50}, calculated by nonlinear regression analysis of each individual concentration-response curve using GraphPad Prism software. n is the number of vessels used.

**Results**

The baseline clinical characteristics of all participants are summarized in the Table. Baseline and NE-induced contraction of vascular lumen diameters did not significantly differ among groups (Table).

**Role of NO Availability and ROS Production on Endothelium-Dependent Relaxation**

Small arteries of EH showed significantly (P<0.001) impaired relaxation to ACh compared with NT (Figure 1A and 1B; Figure S1A), whereas responses to sodium nitroprusside were similar in EH and NT (Figure S1B). In arteries of NT, the relaxation to ACh was significantly blunted by L-NAME (100 μmol/L; Figures 1A and 3) and not affected by ascorbic acid (100 μmol/L; Figure 1A).
By contrast, in vessels of EH, the relaxation to ACh was resistant to L-NAME (Figures 1B and 3). In these vessels, ascorbic acid normalized the endothelium-dependent relaxation and restored the inhibitory effect of L-NAME on ACh (Figures 1B and 3; Figure S1A).

**Influence of COX-1, COX-2 Activity, and TP Receptors on Endothelium-Dependent Relaxation**

In arteries of NT, relaxation to ACh (91.4±2.1%) was not modified by SC-560 (1 μmol/L; 92.3±2.4%) or DuP-697 (1 μmol/L; 92.9±2.7%). By contrast, in vessels of EH, response to ACh (58.2±2.1%), while not modified by SC-560 (59.1±2.6%), was significantly potentiated, but not completely normalized, by DuP-697 (Figure 2A), which also improved the inhibitory effect of L-NAME on ACh (Figures 2A and 3). In vessels from EH, indomethacin (10 μmol/L) improved the relaxing response to ACh to a similar extent than DuP-697 (Figure S2). No further improvement of relaxation to ACh emerged when DuP-697 and SC-560 were simultaneously incubated when compared with DuP-697 alone (Figure S2).

SQ-29548 (1 mmol/L) failed to affect the relaxation to ACh (Figure S2).

**Involvement of NAD(P)H Oxidase, Uncoupled NOS, and XO**

In EH, apocynin (10 μmol/L) moderately, but significantly, improved the relaxation to ACh (Figure 2B) and partly enhanced the inhibition by L-NAME on ACh (Figures 2B and 3). Simultaneous apocynin and DuP-697 infusion normalized the relaxation to ACh (Figure 2B; Figure S1A) and restored the inhibition of L-NAME on ACh (Figure 2B and 3). Similarly, DPI (10 μmol/L) enhanced the relaxation to ACh and, in part, restored the inhibitory effect of L-NAME on ACh (Figure S3). Relaxation to ACh (59.5±1.4%) was unmodified by sepiapterin (100 μmol/L; 59.1±2.5%) or allopurinol (100 μmol/L; 60.3±2.7%).

**Analysis of Vascular Superoxide Anion Generation**

Dihydroethidium analysis revealed a dramatic increase in superoxide anion production in vessels from EH (Figure 4). The superoxide excess was strongly reduced by DuP-697. Apocynin and DPI moderately, but significantly, decreased superoxide generation, whereas SC-560, sepiapterin, or allopurinol was devoid of any effect (Figure 4). Superoxide production was almost abrogated on incubation with ascorbic acid (Figure 4).

**Western Blot Analysis of COX-1 and COX-2**

Small vessels of NT expressed both COX-1 and COX-2 isoforms, but the level of COX-1 expression was higher compared with COX-2 (Figure 5). In small vessels from EH, the COX-1 protein was expressed at a similar extent as in controls. By contrast, a significantly higher expression of COX-2 was detected in arteries of EH compared with that of NT (Figure 5).

**Immunohistochemical Analysis of COX-1 and COX-2**

Appreciable amounts of COX-1 and COX-2 immunostaining were detected throughout the whole wall of arteries of NT and EH. However, different expression patterns of COX-1 and COX-2 were noted when comparing NT with EH. Indeed, in EH vessels, both COX-1 and COX-2 endothelial expression decreased, while a concomitant increase in COX-2 immunostaining was found in the smooth muscle cells. The overall image analysis showed similar percentage levels of COX-1 expression in both NT and EH groups, whereas a predominance of percent COX-2 expression was estimated in EH vessels (Figure 6).

**Discussion**

The results from this study extend to small arteries from subcutaneous fat previous observations that the endothelial dysfunction, known to characterize EH, depends on an increased intravascular ROS generation, which reduces NO availability, which reduces NO availability. In small vessels from EH, the blunted vascular response to ACh was significantly improved by a selective COX-2 inhibitor, which also partly restored the inhibitory effect of L-NAME on ACh. In addition, an augmented COX-2 protein expression in vessels from these patients was documented. Furthermore, immunohistochemistry indicated a marked upregulation of COX-2 mainly in the vascular media.
layer. These data provide the first evidence that, in small arteries isolated from EH, an overexpression and increased activity of COX-2 occur, playing a major role in reducing NO availability.

A possible cross-talk between COX and NO was previously investigated. In line with animal data, human studies demonstrated that intra-arterial infusion of indomethacin or acetylsalicylic acid reversed endothelial dysfunction and restored NO availability in the forearm resistance arteries of EH. Similarly, acute infusion of acetylsalicylic acid improved endothelial dysfunction in the forearm microcirculation of hypercholesterolemic patients or in lower extremity resistance arteries of patients with atherosclerosis. Of note, all these results were obtained with nonselective COX inhibitors. Therefore, while highlighting an active detrimental role of COX on NO-derived endothelium-dependent relaxation, these pioneering investigations have left undetermined the question of which COX isoform was effectively involved. Our present results allow to conclude that COX-2 is the key isoenzyme determining a reduction of NO activity in small vessels from EH. Notably, our proposal is in line with results by Widlansky et al showing that administration of the COX-2 inhibitor celecoxib reversed endothelial dysfunction in the brachial artery of EH. Similarly, celecoxib improved the brachial artery flow-mediated dilation in patients with coronary artery disease.

In essential hypertension or advancing age, the nonselective COX inhibitor indomethacin restored the availability of NO to a similar extent to that obtained with the antioxidant compound ascorbic acid. Furthermore, when indomethacin and ascorbic acid were coinjected, no further amelioration was observed, thus suggesting that ROS production might be the main COX-dependent mechanism responsible for reduced NO availability. Such possibility has been confirmed in this study, documented by a dramatic increase in superoxide production.

Figure 6. Representative images (original magnification ×400) of cyclooxygenase (COX-1; A and C) and COX-2 immunostaining (B and D) in vessels from normotensive controls (NT) and hypertensive patients (EH). Column graphs refer to the image analysis of COX-1 and COX-2 expression in the overall vessel wall of NT and EH. Each column represents the mean of 4 experiments±SEM, OD indicates optical density. *P<0.05.
production in small vessels from EH, which was reduced by the selective COX-2 inhibitor. Thus, our findings provide the first demonstration that COX-2 is the isoform that actively contributes to intravascular superoxide generation in essential hypertension, whereas COX-1 does not seem to be involved. A support to this view comes from both experimental data, identifying COX-2 as a major intravascular source of ROS production, and clinical data, indicating that COX-2 inhibition by celecoxib reduces the plasma concentrations of ox–low-density lipoprotein, a marker of lipid peroxidation.21

Our functional experiments also evidenced that the TP receptor blockade failed to improve the ACh-evoked endothelial response, thus excluding the possibility that the contribution of COX-2 to endothelial dysfunction in this clinical setting might involve the production of contracting prostanoids acting on TP receptors.

The remarkable, but incomplete, reduction of ROS excess by COX-2 inhibition raises the question of whether other sources of ROS, differing from COX-2, might concur to promote ROS generation. To this aim, we investigated the possible role of other recognized vascular enzymatic sources of ROS, such as NAD(P)H oxidase, uncoupled NOS, and XO, by selective pharmacological tools. Apocynin and DPI, but not sepiapterin or allopurinol, partly improved NO availability. Consistently, dihydrothidium staining showed that the NAD(P)H oxidase inhibitors (but not sepiapterin or allopurinol) moderately, but significantly, reduced superoxide generation. These findings allow to propose that, in addition to COX-2 that represents the major ROS source, NAD(P)H oxidase also seems to play a role, albeit minor, in ROS generation in this vascular district of EH. These 2 enzymatic pathways might play a synergistic effect in promoting ROS production, as supported by our functional experiments, showing that, when simultaneously infused, DuP-697 and apocynin normalized the inhibitory effect of L-NAME.

Of note, in addition to being an NAD(P)H oxidase inhibitor, DPI has shown to be a NOS inhibitor, an effect that is clearly not emerging in our experiments. We cannot exclude that, in other experimental conditions, the DPI-mediated NOS blockade might be prevalent.

Notably, the lack of effect of COX-2 pathway in small vessels from normotensive controls agrees with the results from Verna et al, who observed that the pharmacological COX-2 blockade does not affect the endothelial function in healthy volunteers. These data strengthen the concept that COX-2 plays a role in endothelial dysfunction specifically in the pathophysiology of essential hypertension or advancing age, as previously suggested.

Apparently, at variance with results from this study, previous clinical trials raised concerns about an increased cardiovascular risk associated with the COX-2 inhibitor rofecoxib in patients with rheumatoid arthritis or colorectal adenoma. Furthermore, subsequent studies conducted with more selective COX-2 inhibitors, while not confirming such scientific alert, failed to show any cardiovascular protection by coxibs. However, it is important to point out that our experiments were conducted in acute conditions, in EH characterized by endothelial dysfunction, exposed to low risk of cardiovascular disease, and free of atherosclerotic disease.

In conclusion, in small resistance arteries of EH, COX-2 is overexpressed and functionally active, determining the endothelial dysfunction through a reduction of NO availability. COX-2 represents a major source of ROS generation in essential hypertension. In this context, NAD(P)H oxidase also participates, although with a minor role, in promoting superoxide generation in these patients.

**Perspectives**

The reduced NO availability is greatly implicated in the pathogenesis of atherosclerotic disease and cardiovascular events. Therefore, this detrimental action of COX-2 on NO availability represents 1 mechanism, whereby COX-2 may promote atherosclerosis, as documented in animal and human models of vascular disease. Our findings also suggest that the COX-2-mediated pathway might be a possible adjunctive therapeutic target to reverse endothelial dysfunction and, hopefully, to prevent cardiovascular disease in EH.

**Disclosures**

Dr Bernardini was the coordinator of the morphological and immunohistochemical studies. The other authors report no conflicts.

**References**

Hypertension causes premature aging of endothelial function in humans. 


Ruschitzka F. Selective COX-2 inhibition improves endothelial function. 


What Is Relevant?

- We identify for the first time a detrimental action of cyclooxygenase-2 on nitric oxide activity in peripheral small vessels from essential hypertensive patients.
- When considering the importance of endothelial dysfunction as a contributor to the progression of atherosclerotic and cardiovascular events, the cyclooxygenase-2–mediated pathway might be a possible adjunctive therapeutic target in essential hypertensive patients.

Summary

In small resistance arteries, isolated from the subcutaneous adipose tissue of essential hypertensive patients, cyclooxygenase-2 is overexpressed and functionally active, inducing endothelial dysfunction by promoting reactive oxygen species generation which in turn reduces the nitric oxide availability. In this context, nicotinamide adenine dinucleotide phosphate oxidase also participates, although with a minor role, in promoting reactive oxygen species generation in these patients.

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ENDOTHELIAL DYSFUNCTION IN SMALL ARTERIES OF ESSENTIAL HYPERTENSIVE PATIENTS: ROLE OF CYCLOOXYGENASE-2 IN OXIDATIVE STRESS GENERATION.

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**Short Title:** COX-2 and endothelial dysfunction

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Supplemental Methods

Study population.
Essential hypertensive patients were recruited among consecutive patients, referred to the Department of Clinical and Experimental Medicine of the University of Pisa, who underwent laparoscopic surgery for cholecystectomy caused by gallbladder stones or adrenalectomy for a benign and non-functioning adrenal mass over 3.5 cm in size. Normotensive subjects were recruited among patients hospitalized into the Department of Surgery, University of Pisa, to undergo laparoscopic surgery for cholecystectomy. The major inclusion criterion was a clinic blood pressure value (after 10 minutes of rest) >140/90 mmHg, confirmed on 2 separate occasions within 1 month, according to current European Guidelines. Secondary forms of hypertension were excluded by routine diagnostic procedures, including morphological and hormonal investigations when adrenal mass was detected. Other exclusion criteria included clinical or biochemical evidence of thyroid dysfunction, ethanol consumption (more than 60 g per day), dyslipidemia, diabetes mellitus, smoking, body mass index >30 kg/m², renal or liver impairment, and established cardiovascular disease. Patients were never treated for hypertension (n=5) or they had not received any medication for at least 1 month before enrollment in the study (n=9). All women recruited in our population were in postmenopausal status, and no one was receiving hormone replacement therapy.

Preparation, Mounting and Measurements in Small Arteries
Small arteries (150 to 300 µm) were isolated from subcutaneous tissue immediately after biopsy sample procurement and mounted on a pressurized myograph. Vessel segments (~ 2 mm long) were mounted onto 2 glass cannulas, one of which was positioned until the vessel walls were parallel, and equilibrated in physiologic salt solution (mmol/L: NaCl 120, NaHCO₃ 25, KCl 4.7, KH₂PO₄ 1.18, MgSO₄ 1.18, CaCl₂ 2.5, EDTA 0.026, and glucose 5.5) bubbled continuously with 95% air and 5% CO₂ to achieve pH 7.4 at 37°C. Vessels were pressurized at 60 mm Hg. All functional experiments were performed by measuring dilatory responses to agonists in vessels precontracted with norepinephrine (NE, 10⁻⁶ mol/L). The dose of 1 µM NE was chosen after preliminary experiments of concentration-response curves to NE (from 1 nM to 100 µM) in vessels from normotensive subjects and hypertensive patients. Vessels from the two groups showed concentration-dependent contractions to the first four concentrations of NE (from 1 nM to 1 µM), resulting in 50-60% of vessel contraction at the dose of 1 µM, similarly in healthy condition or hypertensive disease.

Detection of vascular superoxide anion generation.
The in situ production of superoxide anion was measured by means of the fluorescent dye dihydroethidium (DHE; Sigma). Three slides per segment were analyzed simultaneously after incubation with antagonists or Krebs solution at 37°C for 30 min. Krebs-HEPES buffer containing 2 µM DHE was then applied onto each section and evaluated under fluorescence microscopy. The percentage of arterial wall area stained with the red signal was evaluated using an imaging software (McBiophotonics Image J; National Institutes of Health, Bethesda, MD).

Western Blot analysis of COX-1 and COX-2.
Specimens of mesenteric arteries were homogenized in radioimmunoprecipitation assay buffer with a polytron homogenizer and centrifuged at 20,000 rpm for 15 min at 4°C. The resulting supernatants were separated from pellets and stored at -20°C. Aliquots of 50 µg of protein were separated by electrophoresis on 8% sodium dodecylsulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane. The blots were then blocked for 1 h with 5% non-fat dried milk in Tween-20 tris-buffered saline (TBS-T) and incubated overnight at room temperature with a primary antibody raised against COX-1, COX-2 (Cayman Chemical Co, Ann Arbor, MI, USA) or β-actin (Sigma-Aldrich, Milano, Italy). After repeated washings with TBS-T, a peroxidase-conjugated secondary antibody was added for 1 h at room temperature. Then, blots were washed and immunoreactive bands were visualized by incubation with chemiluminescent reagents (Immobilon, Millipore, MA, USA) and exposed to
Kodak Image Station 440 (Eastman Kodak Company, Rochester, New York) for signal and densitometric image analysis. To ensure equal loading and accuracy of changes in protein expression, protein levels were normalized to β-actin. In order to verify the specificity of anti-cyclooxygenase antibodies, preliminary experiments were carried out using immunoblot controls purchased from the antibodies supplier (Cayman Chemical). For the COX-1 isoform, ram seminal vesicle microsomes (cat. n. 10004722) were used with the COX-1 monoclonal antibody (cat. n. 160110); for the COX-2 isoform, mouse macrophage microsomal lysate (cat. n. 10004910) were used in SDS-PAGE in conjunction with the COX-2 polyclonal antibody (cat. 160126, Cayman Chemical). Once validated, the primary antibodies were employed for the subsequent experimental procedures.

**Immunostaining of COX isoforms.** 8-micrometer-thick paraffin sections were microwaved, immunostained by primary polyclonal antibodies (anti-COX-1, code No. 160108, and -COX-2 code No. 160126, Cayman Chemical Co, Ann Arbor, MI, USA), treated with biotinylated anti-rabbit immunoglobulins, peroxidase-labeled streptavidin complex (Vector Laboratories, Burlingame, California), and 3,3 diaminobenzidine tetrahydrochloride (DAB) (Dakopatts, Glostrup, Denmark). Negative controls were obtained by substituting the primary antibodies with preimmune rabbit serum. Endogenous peroxidases and avidin-binding activity were assayed by incubating slides with DAB alone or with streptavidin-horseradish peroxidase complex/DAB, respectively. Whole artery sections were examined under light microscope equipped with the digital camera DFC480 (Leica, Cambridge, United Kingdom): the immunoreactive tissue was evaluated as percentage on representative wall microscopic fields with image analysis software (L.A.S. version 4, Leica Mycrosystems; Leica, Cambridge, United Kingdom) and normalized to the total tissue area examined.
TABLE S1. pEC$_{50}$ values of sodium nitroprusside and acetylcholine with or without inhibitors in small vessels from essential hypertensive patients and normotensive controls.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Essential hypertensive patients</th>
<th>Normotensive subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Nitroprusside</td>
<td>6.82±0.12</td>
<td>6.83±0.09</td>
</tr>
<tr>
<td>ACh</td>
<td>5.90±0.05</td>
<td>6.20±0.07 *</td>
</tr>
<tr>
<td>ACh + SC-560</td>
<td>5.84±0.06</td>
<td>6.18±0.08 *</td>
</tr>
<tr>
<td>ACh + Dup-697</td>
<td>5.92±0.10</td>
<td>6.28±0.09 *</td>
</tr>
<tr>
<td>ACh + Apocynin + Dup-697</td>
<td>6.23±0.07</td>
<td>-</td>
</tr>
<tr>
<td>ACh + L-NAME + Apocynin + Dup-697</td>
<td>5.91±0.11</td>
<td>-</td>
</tr>
<tr>
<td>ACh + sepiapterin</td>
<td>5.86±0.06</td>
<td>-</td>
</tr>
<tr>
<td>ACh + allopurinol</td>
<td>5.93±0.08</td>
<td>-</td>
</tr>
</tbody>
</table>

Values as mean ±SEM. pEC$_{50}$ is -logEC$_{50}$, being the concentration of agonist giving half maximal response. *P<0.01. ACh, acetylcholine.
Figure S1. Panel A. Relaxations to acetylcholine in normotensive subjects (NT) and essential hypertensive patients (HT) without or with Apocynin and Dup-697, or Ascorbic acid. Panel B. Relaxations to sodium nitroprusside in NT and HT. Data presented as means ± SEM (n=8). *P<0.001.
**Figure S2.** Relaxations to acetylcholine ± Dup-697, Indomethacin, Dup-697 + SC-560, or SQ-29548 in essential hypertensive patients. Data presented as means ± SEM (n=7). *P<0.001.
Figure S3. Relaxations to acetylcholine ± L-NAME, DPI, or both, in essential hypertensive patients. Data presented as means ± SEM (n=7). *P<0.05.
**Figure S4.** Representative western blot of proteins in human small arteries with antibodies against cyclooxygenases. The blot was cut in two parts: the upper part was first hybridized with anti-COX-1 antibody and, after development, was stripped and probed with anti-COX-2 antibody; the lower part was probed for β-actin and developed.