Increased superoxide anion production by IL-1β impairs nitric oxide mediated relaxation in resistance arteries

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List of non-standard abbreviations:
8-Br-cGMP, 8-Bromoguanosine 3',5'-cyclic monophosphate
IL-1β, interleukin-1β
eNOS, endothelial nitric oxide synthase
iNOS, inducible nitric oxide synthase
L-NAME, Nω-Nitro-L-Arginine Methyl Ester
MRA, mesenteric resistance arteries
NO, nitric oxide
sGC, soluble guanylate cyclase
PEG-SOD, polyethylene glycol superoxide dismutase
SNP, sodium nitroprusside

TNF-α, tumor necrosis factor-α

1400W, N-3-aminomethylbenzylacetamidine

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Abstract

The present study was designed to analyze the effect of long term incubation with interleukin 1β (IL-1β) on endothelium-dependent relaxation in rat mesenteric resistance arteries. Vessels were incubated in culture medium with or without IL-1β (10 ng/ml, 14 h). Changes in lumen diameter were recorded in a pressure myograph. Protein expression, nitrite and superoxide anion (O₂⁻) production were evaluated by either Western blot or immunofluorescence, Griess reaction and ethidium fluorescence, respectively. IL-1β impaired acetylcholine (ACh) and sodium nitroprusside (SNP) vasodilation and increased nitrite and O₂⁻ production and inducible nitric oxide synthase (iNOS), xanthine oxidase and p22^{phox} expression. However, neither endothelial NOS nor soluble guanylate cyclase protein expression were affected by IL-1β treatment. Polyethylene glycol superoxide dismutase partially reversed the impairment of ACh relaxation and abolished the O₂⁻ production observed in IL-1β treated arteries. The impairment of ACh relaxation induced by IL-1β was also partially reversed by the xanthine oxidase inhibitor, allopurinol (1 mM), but not by either the NADPH oxidase inhibitor, apocynine (0.3 mM) or the iNOS inhibitor, 1400W (1 µM). However, all these inhibitors improved the impaired SNP response. The results of the present study demonstrate that long-term incubation with IL-1β induces an impairment of the nitric mediated relaxation in mesenteric resistance arteries through the production of O₂⁻ mainly from xanthine oxidase.
Introduction

Septic shock is characterized by an inflammatory response, hypotension with vasopressor-resistant systemic vasodilatation and the development of multiple organ failure and dysfunction (Das, 2000). This inflammatory response produces a cascade of pro- and anti-inflammatory cytokines and mediators essential for the development of the immune responses to inflammation (Cai et al., 2003). Interleukin-1β (IL-1β) is a proinflammatory cytokine that plays a pivotal role in the inflammatory response underlying septic shock. IL-1β leads to the loss of vascular tone and hypotension primarily through an increase in nitric oxide (NO) production by inducible nitric oxide synthase (iNOS) (Lu and Fiscus, 1999). However, IL-1β also induces alterations in endothelium-dependent relaxations in the earliest (Loughrey et al., 2003) and later (Kessler et al., 1997) phases of sepsis in large arteries. In small vessels, brief exposure of human forearm resistance artery to tumor necrosis factor-α (TNF-α) decreases vasodilatation to acetylcholine (ACh) (Nakamura et al., 2000) and subchronic (three days) in vivo treatment with IL-1β and IL-6 impairs the reduction of perfusion pressure induced by ACh (De Salvatore et al., 2003).

Cytokines may affect endothelial function through a number of signalling mechanisms. Thus, cytokines produce changes in the stability of endothelial NOS (eNOS) mRNA (Dresler and Horning, 1999). In addition, a high concentration of NO downregulates eNOS and soluble guanylate cyclase (sGC) activity (Buga et al., 1993; Papapetropoulos et al., 1996). It has also been reported that IL-1β enhances the generation of superoxide anion (O$_2^-$) in arteries (Visner et al., 1992; Wimalasundera et al., 2003). O$_2^-$ reacts rapidly with NO, producing peroxynitrite (Milstien and Katusic,
1999) which, in turn, reduces NO bioavailability thereby contributing to the impairment of endothelium-dependent relaxation (Cai and Harrison, 2000). Enzymatic sources of $\text{O}_2^-$ production within the vascular wall include NAD(P)H oxidase, xanthine oxidase and NOS (Zalba et al., 2001; Touyz and Schiffrin, 2004; Brandes and Kreuzer, 2005). Furthermore, the nitration of protein tyrosine residues by peroxynitrite may inhibit the enzymes involved in endothelium-dependent relaxation.

Cytokine levels are elevated in most cardiovascular pathologies such as sepsis, hypertension, diabetes and atherosclerosis in which the existence of endothelial dysfunction has also been described (Vila and Salaices, 2005). Disturbances in peripheral vascular resistance are basic contributors to different cardiovascular pathologies. However, there are few studies analyzing the contribution of proinflammatory mediators to the effect of cytokines on endothelium-dependent relaxation in resistance arteries (Vila and Salaices, 2005). The purpose of the present study was, first, to determine the effect of long term incubation with IL-1$\beta$ on endothelium-dependent relaxation of rat mesenteric resistance arteries (MRA) and, second, to analyse the mechanisms involved in the effect of IL-1$\beta$ thoroughly.
Materials and Methods

**Animals and tissue preparation.** Four month-old male Sprague-Dawley rats (Harlam Ibérica, Spain) were used. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The rats were decapitated and the mesenteric arcade was removed and placed in Krebs-Henseleit solution (KHS) with the following composition (in mM): NaCl 112.0; KCl 4.7; CaCl₂ 2.5; KH₂PO₄ 1.1; MgSO₄ 1.2; NaHCO₃ 25.0 and glucose 11.1, maintained at 4°C and continuously gassed with 95% O₂ and 5% CO₂.

**Vessel organ culture.** Segments of third order branches (vascular reactivity, O₂⁻ production and immunofluorescence studies) and second and third order branches (Western Blot and nitrite production studies) of the mesenteric artery were dissected free of fat and connective tissue for the corresponding studies. Vessels were washed three times in Phosphate Buffered Saline supplemented with penicillin (1000 U/ml) and streptomycin (1 mg/ml) (PBS; Pen-Strep). Afterwards, vessels were equilibrated for 1 h in Dulbecco’s modified Eagle medium supplemented with Pen-Strep, 200 mM glutamine and 1% foetal calf serum. All tissue incubations were carried out at 37°C in an atmosphere of 5% CO₂ with a culture incubator. The medium was then replaced with fresh medium with or without 10 ng/ml IL-1β (Roche Diagnostics GmbH, Manheim, Germany) and incubated for 14 h. In some experiments, allopurinol (1 mM), apocynin (0.3 mM), 1400W (1 μM) or L-NAME (100 μM) were added to the incubation medium.
After 14 h incubation, third order branches were transferred to fresh oxygenated KHS at 5ºC for 1 h before mounting in a pressure myograph. Second and third order branches were frozen in liquid nitrogen and kept at –70ºC until the day of the protein expression assay.

This organ culture model was chosen to avoid phenotypic alterations of smooth muscle cells, which can occur in cell culture, and to preserve the in vivo cell-to-cell and cell-to-matrix interactions. Vessels were incubated in culture medium in sterile conditions to assess the effect of IL-1ß on vascular function without the interference of other endotoxin contaminants.

**Pressure myography.** Third order branches of the mesenteric artery were mounted in a small vessel pressure myograph (Danish Myo Tech, Model P100, J.P. Trading I/S, Aarhus, Denmark). Vessels were placed on two glass microcannulae, secured with a surgical nylon suture and adjusted so that the vessel walls were parallel without stretching. Intraluminal pressure was raised to 140 mm Hg and the artery was unbuckled by adjusting the cannulae. The artery was then set to a pressure of 70 mm Hg and allowed to equilibrate for 30 min at 37ºC in KHS gassed with a mixture of 95% O₂ and 5% CO₂. The maximal contractile response was determined by exposure of the vessels to KCl (100 mM). After washing, the tissues were left to equilibrate for 45 min and concentration response curves to ACh (1 nM-10 µM) and sodium nitroprusside (SNP; 0.01 µM-1 mM) were performed in vessels precontracted with 2 µM of phenylephrine. In one set of experiments, the concentration response curves to ACh were performed in the presence of polyethylene glycol superoxide dismutase (PEG-SOD, 200 U/mL; 2 h). In another set of experiments, concentration
response curves to ACh and SNP were performed in arteries incubated overnight (14 h) and throughout the experiment with allopurinol (1 mM), apocynin (0.3 mM), 1400W (1 μM) or L-NAME (100 μM).

In addition, consecutives concentration response curves to the guanosine 3',5'-cyclic monophosphate (cyclic GMP) analogue, 8-Br-cGMP (10 nM-1 mM) and to papaverine (0.1 μM-0.1 mM), were performed in different arteries. At the end of the experiments, some vessels were transferred to a cryomold containing Tissue Tek OCT embedding medium for 20 min (Bayer Química Farmacéutica, Barcelona, Spain), and then immediately frozen in liquid nitrogen for storage at –70º C until O2\textsuperscript{-} measurement.

**Nitrite levels.** NO production was determined by measuring the nitrite contents with the Griess reagent (Green et al., 1982). The absorbance at 540 nm was measured and the nitrite concentration was determined by using a calibration curve of standard sodium nitrite concentrations versus absorbance. Nitrite levels were corrected by mg wet weight.

**Western Blot.** After being homogenized in lysis buffer, tissue samples (25 μg protein) were electrophoretically separated on a 7.5% SDS-PAGE and transferred to polyvinylidene difluoride membranes overnight. Western immunoblot was performed with monoclonal antibody for iNOS and eNOS (1:10000, 1:2500, respectively; Transduction Laboratories, Lexington, UK) and polyclonal antibody for sGC; (1:1000; Alexis Biochemicals, Notttingham, UK) and xanthine oxidase (1:200, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). After washing, membranes were incubated with peroxidase-conjugated secondary antibodies (anti-mouse and anti-
rabbit IgG 1:2000; Transduction Laboratories). Immunocomplexes were detected using an enhanced horseradish peroxidase/luminol chemiluminiscence system (ECL Plus, Amersham International, plc, Little Chalfont, UK). Membranes were subjected to autoradiography (Hyperfilm ECL, Amersham International). Signals on the immunoblot were quantified using a BioRad GS700 and the Molecular Analyst 1.5 software (BioRad). The same membrane was used to determine β-actin expression, using a polyclonal antibody anti-β-actin (1:15000, Sigma Aldrich, Spain).

**Immunofluorescence.** Arterial segments were fixed with 4% phosphate buffered paraformaldehyde (pH=7.4) for 1 h and washed in three changes of phosphate buffered saline solution (PBS, pH=7.4). After clearing, arterial segments were placed in PBS containing 30% sucrose, transferred to a cryomold containing Tissue Tek OCT embedding medium (Sakura Finetek Europe, The Netherlands) and frozen in liquid nitrogen. Tissues were kept at −70°C until the day of the experiments. Frozen transverse sections (14 µm) were cut onto gelatine-coated slides and air-dried for at least 60 min. After blockade, sections were incubated with a mouse monoclonal antibody against iNOS (1:100), xanthine oxidase (1:1000, Neomarkers, Fremont, CA, USA) or a goat polyclonal antibody against the NADPH oxidase subunit p22phox (1:100, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) in PBS containing 2% BSA for 1 h at 37°C in a humid box. After washing, rings were incubated with the secondary antibodies, a donkey anti-mouse or anti-goat IgG conjugated to Cy™3 (Jackson Immunoresearch Laboratories, Inc., West Grove, PA, USA) at a dilution 1:200 for a further 1 h at 37°C in a humid box. After washing, immunofluorescent signals were viewed using an inverted Leica TCS 4D confocal laser scanning
microscope with oil immersion lens (x40). Cy<sup>TM</sup>3 labelled antibody was visualized by excitation at 568 nm and detection at 600-700 nm.

The specificity of the immunostaining was evaluated by omission of the primary antibody and processed as above. Under these conditions, no staining was observed in the vessel wall in any experimental situation.

**Measurement of O<sub>2</sub><sup>-</sup> production.** The oxidative fluorescent dye dihydroethidium was used to evaluate production of O<sub>2</sub><sup>-</sup> in situ, as described previously (Miller et al., 1998). Hydroethidine permeates cells freely and, in the presence of O<sub>2</sub><sup>-</sup>, is oxidized to ethidium bromide which is trapped by intercalation with DNA. Ethidium bromide is excited at 488 nm and has an emission spectrum of 610 nm. Frozen tissue segments were cut into 14 µm thick sections and placed on a glass slide. Serial sections were equilibrated under identical conditions for 30 minutes at 37°C in Krebs-HEPES buffer (in mM: NaCl 130, KCl 5.6, CaCl<sub>2</sub> 2, MgCl<sub>2</sub> 0.24, HEPES 8.3, glucose 11, pH 7.4). Fresh buffer containing dihydroethidium (2 µM) was applied topically onto each tissue section, cover-slipped and incubated for 30 minutes in a light-protected humidified chamber at 37°C, and then viewed by fluorescent laser scanning confocal microscope (Leica TCS 4D equipped with a krypton/argon laser), using the same imaging settings in each case. Fluorescence was detected with a 568 nm long-pass filter. In some experiments, parallel sections were incubated with either PEG-SOD (500 U/ml), allopurinol (1 mM) or apocynin (0.3 mM). For fluorescence quantification, four areas per ring were sampled for each experimental condition. All measurements were conducted blind. The integrated optical densities in the target region were calculated.
**Drugs and solutions.** Drugs used were: acetylcholine chloride, 8-bromoguanosine 3',5'-cyclic monophosphate, dihydroethidium, 4-hydroxy-3-methoxyacetophenon, 4-hydroxypyrazolo[3,4-d]pyrimidine, N-3-aminomethylbenzylacetamidine, N\textsuperscript{w}-nitro-L-arginine methyl ester, polyethylene glycol superoxide dismutase, phenylephrine hydrochloride, sodium nitroprusside, superoxide dismutase and papaverine hydrochloride (Sigma Chemical Co., St. Louis, MO, USA); interleukin-1\textbeta (Roche Diagnostics GmbH, Manheim, Germany).

**Data analysis and statistics.** Relaxation responses were expressed as a percentage of the increase in the internal diameter obtained during the contraction to phenylephrine and calculated from the following expression:

\[
\% \text{ Relaxation} = \frac{(LD_1-LD_2)}{(LD_3-LD_2)} \times 100 \quad \text{equation 1}
\]

Where LD\textsubscript{1} is the lumen diameter achieved by a particular concentration of agonist; LD\textsubscript{2} is the lumen diameter achieved by preconstriction with phenylephrine in the absence of agonist and LD\textsubscript{3} is the lumen diameter when the artery is fully relaxed.

Protein expression data are expressed as the ratio between signals on the immunoblot corresponding to eNOS, iNOS, xanthine oxidase, sGC and β-actin.

All results are expressed as mean ± S.E.M. The number (n) of rats is indicated in the figure legends. The dependency of relaxation response on concentration and treatment was assessed by a two-way (concentration, treatment) analysis of variance (ANOVA) with repeated measures on the concentration factor. When the effect of a treatment was significant, differences between pairs of curves (treatments) were analysed using appropriate contrasts within the ANOVA test. The possible interaction
between concentration and treatment was also considered. For Western blot and nitrite production experiments the differences between treatments were analysed by one-way ANOVA. For $O_2^-$ production the difference between treatments was analyzed either by one-way ANOVA using Tukey test for multiple comparisons or by paired Student’s t-test as appropriate. A value of $p<0.05$ was considered significant.

Data analysis was carried out with the SAS/STAT® release 8.01 statistical package (SAS Institute Inc., Cary, NC).
Results

Effect of IL-1β on endothelium-dependent and independent relaxation. ACh (1 nM-10 µM; Fig. 1A) and SNP (1 nM-1 mM; Fig. 1B) respectively induced endothelium-dependent and independent, concentration-related relaxations of phenylephrine pre- contracted rat MRA. Incubation (14 h) of the vessels in culture medium either in the absence or in the presence of IL-1β (10 ng/ml) reduced the vasodilatation induced by both ACh and SNP. However, the impairment of the ACh and SNP induced relaxations was greater in the presence than in the absence of IL-1β (Fig.1A and 1B). The non selective NOS inhibitor, L-NAME (100 µM), abolished the ACh induced relaxation in control arteries incubated in culture medium. However, L-NAME did not modify the concentration response curve when the arteries were incubated with IL-1β (Fig. 1C). The incubation medium, with or without IL-1β, did not alter the papaverine (0.1 µM-0.1 mM, Fig. 1D) or 8-Br-cGMP (10 nM-1 mM; Fig. 1E) induced vasodilatations.

Effect of IL-1β on eNOS and sGC protein expression. Analysis of Western blot data revealed that neither eNOS (Fig. 2A) nor sGC (Fig. 2B) protein expression was modified after the incubation of MRA in culture medium (14 h) in either the absence or presence of IL-1β (10 ng/ml).

Effect of IL-1β on NO production and iNOS protein expression. Fourteen hours incubation in culture medium induced a slight production of nitrite that was further increased by the incubation with IL-1β (Fig. 3A). Freshly isolated or culture medium incubated arteries did not show any expression of iNOS. However, the incubation with IL-1β strongly induced the expression of this protein (Fig. 3B).
Immunofluorescence studies showed that iNOS was localized in endothelial and smooth muscle cells only after IL-1β incubation (Fig. 3C).

**Effect of IL-1β on O$_2^-$ production.** After 14 h incubation in culture medium, the arteries showed a fluorescence signal that was further increased in the presence of IL-1β (10 ng/ml), reflecting an increase in O$_2^-$ production (Fig. 4A). Ethidium fluorescence was notable in the three layers of the vascular wall. The permeable O$_2^-$ scavenger, PEG-SOD (500 U/ml), reduced the observed fluorescence in both the control conditions and after IL-1β treatment (Fig. 4A). Incubation with allopurinol (1 mM), a xanthine oxidase inhibitor, reduced the increase of ethidium fluorescence induced by IL-1β (Fig. 5). However, the NADPH oxidase inhibitor, apocynin (0.3 mM) only produced a slight reduction of the IL-1β induced fluorescence that did not reach statistical significance (Fig. 5).

**Effect of inhibitors of O$_2^-$ production and iNOS in the effect of IL-1β on vascular function.** PEG-SOD (200 U/mL) improved the observed impairment of ACh relaxation in arteries incubated either in the presence or in the absence of IL-1β. However, the ACh response was still impaired when compared to the responses observed in freshly isolated arteries (Fig. 4B). The effect of IL-1β on ACh mediated vasodilatation was also reduced by allopurinol (1 mM; Fig. 6A) but not by apocynin (0.3 mM; Fig. 6C) or the iNOS selective inhibitor, 1400W (1 µM; Fig. 6E). However, the impairment of SNP relaxation induced by IL-1β was reduced by the three inhibitors (Figs. 6B, 6D and 6F). In addition, both allopurinol (Figs. 6A and 6B) and apocynine (Figs. 6C and 6D) but not 1400W (Figs. 6E and 6F) improved the observed impairment of ACh and SNP responses in arteries incubated without IL-1β.
Effect of IL-1β on p22phox and xanthine oxidase expression. IL-1β increased the fluorescence corresponding to xanthine oxidase (Fig. 7A) and p22phox (Fig. 7B) mainly in the media layer of the mesenteric arteries. The increase of xanthine oxidase protein expression after IL-1β treatment was confirmed by Western blot analysis (Fig. 7A).
Discussion

Bacterial products involved in endotoxic shock are associated with reduced endothelial vasodilator responses (Brandes et al., 1999; Piepot et al., 2000; Hernanz et al., 2004; Virdis et al., 2005). During the onset of experimental sepsis, the increase observed in serum cytokines levels (Riedmann et al., 2003, Virdis et al., 2005), could be responsible for the reported endothelial dysfunction. In a previous study (Briones et al., 2005), we reported an impairment of endothelial-dependent relaxation by IL-1β in MRA. Here we confirm these results and focus on the elucidation of the mechanisms involved. The present study demonstrate that prolonged incubation with IL-1β impairs the NO mediated relaxation in the MRA through the production of O$_2$^- synthesized mainly by xanthine oxidase.

ACh induced an endothelial-dependent relaxation in MRA that was dependent on NO release in accordance with previous studies (Virdis et al., 2005). Incubation with IL-1β decreased the ACh mediated relaxation similarly in the presence and in the absence of L-NAME suggesting that the impairment of ACh relaxation by IL-1β could be due to a decrease on NO availability. Alterations in the synthesis and/or effect of NO could explain the depressor effect induced by IL-1β in ACh vasodilatation. In a co-culture system, IL-1β-treated smooth muscle cells decreased the eNOS protein expression in endothelial cells (De Frutos et al., 1999). Furthermore, TNF-α impaired the stability of eNOS mRNA (Yoshizumi et al., 1993). Nevertheless, we did not find changes in eNOS protein expression after IL-1β treatment. Vasodilator responses to SNP but not to papaverine were impaired by IL-1β, suggesting that the effect of IL-1β is selective for NO mediated relaxation. Nitric oxide produces vasodilatation, among
others, by stimulating sGC which enhances cGMP levels in vascular smooth muscle.

In our experimental conditions, we did not find changes in either sGC expression or the vasodilatation mediated by the cGMP analogue, 8-Br-cGMP. In agreement, Tsuchida et al. (1994) reported unaltered responses to 8-Br-cGMP after lipopolysaccharide.

Inflammatory responses are associated with increases in $\text{O}_2^-$ production (Guzik et al., 2003; Salvemini et al., 2003). The newly formed $\text{O}_2^-$ can scavenge NO, effectively reducing the bioavailability of endothelium-derived NO and thus leading to vascular dysfunction (Hamilton et al., 2002). Our results in MRA show that IL-1$\beta$ greatly enhanced the formation of $\text{O}_2^-$. In this sense, an increase in $\text{O}_2^-$ production after lipopolysaccharide has been reported in rat aorta (Brandes et al., 1999) and middle cerebral artery (Hernanz et al., 2003). Based on these findings, we hypothesized that an increase in NO breakdown induced by an excess of $\text{O}_2^-$ could be responsible for the endothelial dysfunction observed after IL-1$\beta$ treatment. The fact that the membrane permeable SOD analogue, PEG-SOD reverted both the impairment of the ACh vasodilatation and the increase in $\text{O}_2^-$ production, support this hypothesis.

Incubation medium alone produced effects which were similar, albeit less severe, to those of IL-1$\beta$. Thus, in the absence of IL-1$\beta$ we observed a slight increase in $\text{O}_2^-$ production, that would correlate with the rightward shift of the concentration-response curve to ACh compared to freshly isolated vessels. Accordingly, PEG-SOD partially revert the impairment of ACh vasodilatation produced by the incubation medium. It is possible that either endothelial damage or changes in the signalling
pathway for ACh are occurring. However, mediators other than $O_2^-$, may participate in the impairment of ACh responses observed in control conditions. Thus, a remarkable production of contractile prostanoids was observed in arteries incubated with culture medium (Briones et al., 2005). These prostanoids may participate on the impairment of endothelium dependent relaxation (Hernanz et al., 2004), although its role has not been addressed in the present study. The origin of the alterations induced by culture medium is unclear. The prolonged exposure to very small amounts of lipopolysacharide in nominally sterile media (McKenna et al., 1994) could be implicated. Furthermore, incubation alone may elicit vascular synthesis of cytokines that can participate in $O_2^-$ production (Newman et al., 1996). In this line of evidence, Wylam et al. (2001) reported that incubation medium *per se* may produce alterations in vascular responses that are in addition to those induced by lipopolysacharide.

NADPH oxidase is an important source of vascular $O_2^-$ (Griendling et al., 2000; Zalba et al., 2001; Touyz and Schiffrin, 2004; Brandes and Kreuzer, 2005). Apocynin, a NADPH oxidase inhibitor, improved both the ACh and the SNP responses in vessels incubated in the absence of IL-1$\beta$ supporting the participation of NADPH oxidase in the basal $O_2^-$ production. The participation of NADPH oxidase in both $O_2^-$ generation by TNF-$\alpha$ (Ungvari et al., 2003) and in the altered vasoconstriction by lipopolysacharide (Hernanz et al., 2003) have been described. In our study, IL-1$\beta$ increased p22$^{phox}$ expression and $O_2^-$ production, in accordance with the results obtained in rat aorta after lipopolysacharide treatment (Brandes et al., 1999), and reversed the impairment of the SNP induced vasodilatation. However, apocynin was
unable to modify the effects of IL-1β on ACh relaxation discarding the participation of NADPH oxidase on this effect.

Xanthine oxidase can also be involved in \( \text{O}_2^- \) production (Cai and Harrison, 2000). The expression of this enzyme was increased by IL-1β incubation, as previously reported after lipopolysaccharide treatment (Brandes et al., 1999). The participation of this enzyme in the observed endothelial dysfunction after IL-1β treatment was confirmed by the findings that allopurinol: 1) decreased the impairment in ACh relaxation, 2) reversed the impaired SNP relaxation, and 3) reduced \( \text{O}_2^- \) production. In middle cerebral arteries, we also showed that allopurinol decreased the effect of lipopolysaccharide on serotonin responses (Hernanz et al., 2003). In contrast, in hyperhomocysteineemic coronary arteries, an increase in the generation of \( \text{O}_2^- \), was unaffected by allopurinol (Ungvari et al., 2003). In control conditions, the impairment of ACh and SNP vasodilatation was also reduced by allopurinol supporting the participation of this enzyme on the \( \text{O}_2^- \) generation also in these experimental condition. It is difficult to dilucidate if the improvement by allopurinol on the impaired ACh responses by IL-1β is due to the inhibition of either basal or IL-1β stimulation of \( \text{O}_2^- \) production. The fact that allopurinol decreases ethidium fluorescence after IL-1 β, suggest that the effect of allopurinol on ACh responses could be due to the decrease of both basal and IL-1 β stimulated \( \text{O}_2^- \) production.

IL-1β incubation increases iNOS protein expression and nitrite production, as previously described in MRA from young and old rats (Briones et al., 2005). The involvement of iNOS-generated NO in the impairment of the endothelium-dependent relaxations induced by IL-1β or lipopolysaccharide has been demonstrated in rabbit
carotid (Kessler et al., 1997) and rat middle cerebral arteries (Hernanz et al., 2004). When IL-1β was present in the culture medium, 1400W recovered SNP induced relaxation but was without effect on ACh relaxation. It is possible that NO produced by SNP or by cytokines could regulate the formation of reactive oxygen species through the modulation of O$_2^-$ generating systems such as NADPH oxidase, as suggested (Kaur et al., 2004). Our results point in this direction since apocynin also improved the impairment of SNP induced relaxation induced by IL-1β.

The finding that all inhibitors improved the impaired SNP vasodilatation but only allopurinol was able to slightly improve the ACh relaxation in vessels incubated with IL-1β is intriguing. NO from eNOS must cross two plasma membranes and the space between the endothelium and smooth muscle before it can activate sGC. However, SNP releases NO directly in the smooth muscle cells that, in turn, would be closer to the sGC than is ACh-derived NO. If so, endothelial NO would be more susceptible to inactivation by O$_2^-$ produced in smooth muscle and endothelial cells than NO from SNP, which would be easily inactivated by the O$_2^-$ present in the smooth muscle cells. This putative mechanism would help to explain the better recovery of SNP induced response compared to relaxations induced by ACh when the amount of O$_2^-$ decreases. This hypothesis was also suggested by Gunnett et al. (2000) in carotid arteries from IL-10 -/- mice to explain the different effects of lipopolysacharide on ACh and SNP vasodilatations despite an increase in O$_2^-$ production.

In conclusion, our results indicate that long-term incubation with IL-1β produces an excess of O$_2^-$ mainly synthesized by xanthine oxidase that plays a crucial role in the development of impaired NO mediated relaxation in MRA. The newly formed O$_2^-$
could scavenge NO producing peroxynitrite and thereby reduce the bioavailability of the endothelium derived NO that would, in turn, participate in the endothelial dysfunction. However, the effects of IL-1β cannot be solely explained on the basis of decreased NO bioavailability. Cytokines act synergistically on the initiation of the inflammatory cascade, leading to the expression of other cytokines, cytokine receptors and genes that synthesize several mediators all of which can affect vascular function (for review see Vila and Salaices, 2005). Several cardiovascular diseases such as congestive heart failure, atherosclerosis, septic shock, diabetes and hypertension are associated to elevated levels of pro-inflammatory cytokines. Our results support the participation of cytokines, such as IL-1β, as mediators of the endothelial dysfunction that is widely described in these vascular pathologies.

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Footnotes

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Figure legends

Fig. 1. Concentration response curves to acetylcholine (A), sodium nitroprusside (B) acetylcholine in the presence or absence of L-NAME (100 µM) (C), papaverine (D) and 8-Br-cGMP (E) in phenylephrine pre-contracted rat mesenteric resistance arteries freshly isolated (Fresh) or incubated (14 h) in culture medium without (Control) or with interleukin 1β (IL-1β, 10 ng/ml). Results are the mean ± S.E.M of 6-13 animals. **p<0.01; ***p<0.001 by two way ANOVA.

Fig. 2. Representative blot and densitometric analysis of endothelial nitric oxide synthase (eNOS, A) and soluble guanylate cyclase (sGC, B) protein expression in mesenteric resistance arteries freshly isolated (Fresh) or incubated (14 h) in culture medium without (Control) or with interleukin-1β (IL-1β, 10 ng/ml). The expression of β-actin as a loading control is also shown. Results are the mean ± S.E.M of 5-7 animals.

Fig. 3. (A) Levels of nitrite measured in culture medium of mesenteric resistance arteries incubated (14 h) in the absence (Control) or in the presence of interleukin-1β (IL-1β; 10 ng/ml). (B) Representative blot and densitometric analysis of iNOS protein expression in mesenteric resistance arteries freshly isolated (Fresh) or incubated (14 h) in culture medium without (Control) or with IL-1β. The expression of β-actin, as a loading control is also shown. Results are the mean ± S.E.M., n=6 animals.**p<0.01 vs Control; by one-way ANOVA. (C) Representative photomicrographs of iNOS immunofluorescence in transversal sections of rat mesenteric resistance arteries freshly isolated (Fresh) or incubated (14 h) in
culture medium without (Control) or with IL-1β. iNOS was labelled with a secondary antibody conjugated to Cy3 appearing as red. Natural autofluorescence of the internal elastic lamina (IEL), appearing as green, is a means way to delimitate intima and media layers. EC: endothelial cells; SMC: smooth muscle cells; IEL: internal elastic lamina. \( n=4 \) animals. Image size: 256 x 256 \( \mu m \).

**Fig. 4.** (A) Representative fluorescence photomicrographs and quantification of confocal microscopic sections of rat mesenteric resistance arteries freshly isolated (Fresh) or incubated (14 h) in culture medium without (Control) or with interleukin 1β (IL-1β,10 ng/ml) in the absence or in the presence of PEG-SOD (500 U/ml). Vessels were labeled with the oxidative dye hydroethidium, which produces a red fluorescence when oxidized to ethidium bromide by superoxide anion. AC, adventitial cells; EC, endothelial cells; SMC, smooth muscle cells; IEL, internal elastic lamina; Number of animals=6. Image size: 256 x 256 \( \mu m \). \#p<0.05; ### \( p<0.001 \) vs Fresh; * \( p<0.05 \) vs Control; †\( p<0.05 \); ‡\( p<0.01 \) vs PEG-SOD non incubated vessels by one way ANOVA followed by Tuket test. (B) Influence of PEG-SOD (200 U/ml) on concentration response curve to acetylcholine in phenylephrine pre-contracted rat mesenteric resistance arteries incubated (14 h) in culture medium without (Control) or with interleukin 1β (IL-1β,10 ng/ml). The response to ACh on non-incubated (Fresh) arteries is also shown. Results are the mean ± S.E.M. of 6-11 animals. *\( p<0.05 \); **\( p<0.01 \); ***\( p<0.001 \) by two way ANOVA.
**Fig. 5.** Representative fluorescence photomicrographs (A) and quantification (B) of confocal microscopic sections of rat mesenteric resistance arteries incubated (14 h) in culture medium with interleukin 1β (IL-1β, 10 ng/ml) in the absence or in the presence of allopurinol (1 mM) or apocynin (0.3 mM). Vessels were labeled with the oxidative dye hydroethidine, which produces a red fluorescence when oxidized to ethidium bromide by superoxide anion. Number of animals=5. Image size: 256 x 256 μm. *p<0.05 vs Control by paired Student’s t-test

**Fig. 6.** Influence of allopurinol (1 mM, A, B), apocynin (0.3 mM C, D) and 1400W (1 μM, E, F) on the concentration response curves to acetylcholine (A, C, E) and sodium nitroprusside (B, D, F) in phenylephrine pre-contracted rat mesenteric resistance arteries incubated (14 h) in culture medium without (Control) or with interleukin 1β (IL-1β, 10 ng/ml). Results are the mean ± S.E.M. of n= 6-12 animals. **p<0.01; ***p<0.001 by two way ANOVA.

**Fig. 7.** (A) Representative photomicrographs and blot and densitometric analysis of xanthine oxidase (XO) protein expression; (B) representative photomicrographs of p22phox immunofluorescence in mesenteric resistance arteries incubated (14 h) in culture medium without (Control) or with interleukin 1β (IL-1β, 10 ng/ml). The expression of β-actin as loading control is also shown. Arrowheads show positive staining. Number of animals=3. IEL: internal elastic lamina. Image size: 256 x 256 μm. *p<0.05 by Student’s t-test.