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RESEARCH PAPER

Nitric oxide, derived from inducible nitric oxide synthase, decreases hypoxia inducible factor-1α in macrophages during aspirin-induced mesenteric inflammation

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Background and purpose: Nitric oxide (NO) modulates expression of hypoxia inducible factor-1 (HIF-1), a transcription factor regulating function of myeloid cells. Here, we have assessed the role played by NO, formed by inducible NOS (iNOS), in the inflammation induced by aspirin in the gut, by modulating HIF-1 activity.
Experimental approach: The role of iNOS-derived NO on leucocyte–endothelial interactions induced by aspirin was evaluated by intravital microscopy in mesenteric venules of rats pretreated with selective iNOS inhibitors, 1400W or L-N6-(1-iminoethyl)-lysine. NO was localized by fluorescence microscopy, using DAF-FM. iNOS, HIF-1α and CD36 were localized by immunohistochemistry.
Key results: Leucocyte–endothelial interactions increased at 6 h and returned to normal levels 24 h after aspirin administration. Numbers of migrated leucocytes were similar between 6 and 24 h after aspirin. iNOS expression and iNOS-derived NO synthesis were observed in leucocytes of the mesentery of aspirin-treated rats. Blockade of iNOS activity in aspirin-treated rats: (i) did not modify leucocyte infiltration at 6 h, but reduced the number of polymorphonuclear leucocyte and increased that of macrophages at 24 h; (ii) increased HIF-1α immunostaining in macrophages of the mesentery; and (iii) prevented the decrease in CD36 immunostaining induced by aspirin in these cells.
Conclusions and implications: NO, associated with acute gut inflammation induced by aspirin, diminished HIF-1α stabilization in macrophages. Early inhibition of iNOS-derived NO synthesis, by increasing the activity of HIF-1 in these cells, may accelerate the clearance of leucocytes.
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Keywords: HIF-1; hypoxia; iNOS; aspirin; inflammation; CD36

Abbreviations: eNOS, endothelial NOS; HIF-1, hypoxia inducible factor-1; iNOS, inducible NOS; L-NIL, L-N6-(1-iminoethyl)-lysine; PMN, polymorphonuclear leucocyte

Introduction

Gastrointestinal toxicity induced by non-steroidal anti-inflammatory drugs is associated with inflammation, a complex process of vascular and cellular events that involve the sequential release of mediators and the recruitment of circulating leucocytes, which become activated at the inflammatory site and release further mediators. The acute inflammatory processes are commonly self-limiting, and the resolution of inflammation depends on apoptosis of inflammatory cells, mainly neutrophils, and their subsequent clearance by phagocytes, especially macrophages (Serhan and Savill, 2005).

Nitric oxide (NO) is one of the effector molecules released by activated leucocytes. NO formed by inducible NOS (iNOS) is associated with an inflammatory response, and in contrast to the well-known anti-inflammatory effects of NO derived from endothelial NOS (eNOS), its role in inflammation is still controversial. Both anti-inflammatory and pro-inflammatory actions have been described (Kubes, 2000; Laroux *et al.*, 2000;

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Hickey, 2001), mainly depending on the acute or chronic nature of the process, the phase analysed, the enzymatic/cellular source responsible for NO synthesis or even the tissues affected by the inflammation (Kubes, 2000; Laroux *et al.*, 2000; Hickey, 2001)

In recent years, modulation by NO of the activity of the transcription factor hypoxia inducible factor-1 (HIF-1) has been reported in cultured cells (Sogawa *et al.*, 1998; Kimura *et al.*, 2000; Yin *et al.*, 2000; Sandau *et al.*, 2001; Agani *et al.*, 2002; Hagen *et al.*, 2003; Mateo *et al.*, 2003). NO seems to play a dual role in HIF-1α stabilization, depending on its concentration and oxygen levels. High concentrations of NO induce HIF-1α stabilization in both normoxia and hypoxia, while low concentrations of NO induced HIF-1α destabilization in hypoxia. At an inflammatory focus, HIF-1 plays a role in the adaptation of recently extravasated leucocytes to the new tissue environment (Walmsley *et al.*, 2005), and thus HIF-1 modulates the functioning of myeloid cells as effectors of the innate immune system (Murdoch *et al.*, 2005; Walmsley *et al.*, 2008; Zinkernagel *et al.*, 2007). Considering that many pathways in the inflammatory microenvironment (hypoxia, NO or several cytokines) have been shown to affect HIF-1α levels, the present study aimed to determine the specific role of NO in the stabilization of HIF-1α in inflammatory cells of the mesentery, and the consequences of this regulation for the evolution of the inflammation. Our results showed that iNOS-derived NO synthesis in mesenteric macrophages of aspirin-treated rats prevented HIF-1α stabilization in these cells and delayed the clearance of the mesenteric polymorphonuclear leucocyte (PMN) infiltrate caused by aspirin.

Methods

Animals

Male Sprague-Dawley rats (200–250 g) deprived of food for 16–20 h received a single dose of aspirin (150 $mg \cdot kg^{-1}$, p.o.; Sigma Chemical, St Louis, MO, USA) or vehicle (carboxymethyl cellulose 1%). Experiments were performed 6 or 24 h later. Some rats received a single dose of the selective iNOS inhibitor 1400W (5 $mg \cdot kg^{-1}$, s.c.; Alexis Corporation, San Diego, CA, USA), L-N6-(1-iminoethyl)-lysine (L-NIL; 5 $mg \cdot kg^{-1}$, i.p.) or saline (1 $mL \cdot kg^{-1}$), 10 min before aspirin. All protocols complied with the European Community's guidelines for the use of experimental animals.

Intravital microscopy

Leucocyte–endothelial cell interactions were evaluated by intravital microscopy. The details of the experimental preparation have been described previously (Alvarez *et al.*, 2006). In brief, after rats were anaesthetized with sodium pentobarbital (65 $mg \cdot kg^{-1}$, i.p.) a midline abdominal incision was made, and a segment of the mid-jejunum was exteriorized and placed on a transparent pedestal for tissue transillumination. A selected loop of the exposed mesentery was continuously superfused (2 $mL \cdot min^{-1}$) with bicarbonate-buffered saline (in mM: NaCl, 131.9; KCl, 4.7; $MgSO_4$, 1.2; $NaHCO_3$, 20; pH 7.4, 37°C) and observed through an orthostatic microscope equipped with a video camera. Images were captured on videotape for playback analysis (final magnification of the video screen was ×1300).

The mesentery was left to stabilize for a period of 30 min, and images of three unbranched mesenteric venules (with diameters of between 25 and 40 μm) were recorded for a period of 5 min per venule. The numbers of rolling, adherent and emigrated leucocytes were determined off-line during playback analysis of videotaped images. Rolling leucocyte flux was assessed by counting the number of leucocytes passing a reference point in the vessel per min. Leucocyte rolling velocity was calculated by measuring the time required for a leucocyte to traverse 100 μm of the venule and was expressed as $\mu m \cdot s^{-1}$. A leucocyte was considered to be adherent to the venular endothelium if it remained stationary for a period equal to or exceeding 30 s. Adherent cells were expressed as the number of white blood cells per 100 μm of venule. Leucocyte emigration was evaluated as the total number of interstitial leucocytes per field. Systemic arterial blood pressure, venular diameters and centreline red blood cell velocity were evaluated on-line, and venular blood flow and venular wall shear rate (γ) were calculated as previously described (Alvarez *et al.*, 2006).

Imaging of NO synthesis by fluorescence microscopy

NO synthesis was visualized with the cell permeable fluorescent precursor, 4-amino-5-methylamino-2′,7′-difluorofluorescein (DAF-FM) diacetate as previously reported (Quintana *et al.*, 2004). Inside cells, this is hydrolysed by cytosolic esterases to the non-permeable DAF-FM. In the presence of NO and oxygen, the relatively non-fluorescent DAF-FM is converted into the highly fluorescent and photostable triazole form, DAF-FM T, whose fluorescence intensity is directly proportional to the NO concentration. The spectra of the adduct of DAF-FM is independent of pH, and the detection limit of NO by this fluorochrome is 3 nM (Nagano and Yoshimura, 2002).

Tissue staining and microscopy visualization

Three mesenteric windows were extracted from each rat, cleared of surrounding fat and were pre-incubated (30 min, 37°C) with carbogenated Krebs containing vehicle (in mM: HEPES, 2; L-arginine, 0.3; NaCl, 118; KCl, 4.8; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $CaCl_2$, 1; glucose 200), or the NO synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME, 1 mM). Afterwards, both DAF-FM (1 μM) and the vital nuclear staining fluorochrome, Hoechst 33342 (1 $\mu g \cdot mL^{-1}$) were added and maintained for 30 min at 37°C. After rinsing, whole-mount preparations were mounted in a tissue slice chamber in carbogenated Krebs solution, and were observed with an inverted research microscope Ix81 motorised (Olympus Europe, Tokyo, Japan). Images were acquired by a black-and-white camera OBS Hamamatsu (Olympus). Basal autofluorescence was detected in a sample non-treated with DAF-FM, and the microscopy settings were adjusted to produce the optimum signal-to-noise ratio. In order to compare fluorescence signals between different preparations, settings were fixed for all samples from the same animal. To quantify the NO signal in leucocytes of the mesentery, we proceeded to identify them from the bright field image, and the signal was

calculated after subtracting the background signal. Fluorescence analysis was performed using cell^R software (Olympus).

Immunohistochemical studies for iNOS, HIF-1α or CD36

Mesenteric windows were extracted, cleared of surrounding fat, whole mounted on gelatin-coated slides, fixed for 10 min with paraformaldehyde (4% in PBS pH 7.4), washed with PBS and immersed in methanol (–20°C) for 5 min. After antigen retrieval with α-chymotrypsin (Sigma Chemical Co.) and blocking (10% goat serum, 1% BSA), specimens were incubated with a mouse monoclonal anti-HIF1α (Ab-4, Laboratory Vision Neomarkers, Westinghouse, CA, USA; 1:100, 4°C, overnight), a mouse monoclonal anti-CD36 (Abcam, Cambridge, UK, 1:100, 4°C, overnight) or a rabbit polyclonal anti-iNOS antibody (BD Transduction Laboratories, Erembodegem, Belgium; 1:100, 4°C, overnight). A horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody HRP conjugate (Dako, Glostrup, Denmark; 1:100) was used as secondary antibody and was incubated for 1 h at room temperature to detect both HIF-1α and CD36. To detect the anti-iNOS antibody, we used the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA). In some experiments, mesenteric macrophages were identified by applying an analogous protocol to a second sample from the same animal, but changing the primary antibody for a mouse monoclonal anti-macrophage + granulocyte antibody (OX41, Abcam, 1:400), or a mouse monoclonal antibody against an ED2-like antigen (HIS36, eBioscience, San Diego, CA, USA; 1:100). In all cases, tissues were finally incubated with 3.3-diaminobenzidine (DAB) Enhanced Liquid Substrate System for Immunohistochemistry (Sigma Chemical) and counterstained with haematoxylin. The preparation was subsequently observed under a clear field microscope (40×), and the infiltrated leucocytes were counted (number per 3.6 mm^2) and classified morphologically into PMNs and macrophages by an observer who was unaware of the previous treatment. The specificity of the immunostaining was routinely confirmed by the absence of staining in analogous tissue samples in which either the primary or the secondary antibodies were omitted.

Statistical analysis

Data are expressed as mean ± SEM, and have been compared using one-way ANOVA with a Newman–Keuls *post hoc* correction for multiple comparisons, or a *t*-test, when appropriate. A P value < 0.05 was considered to be statistically significant.

Materials

Aspirin, L-NIL, L-NAME and sodium pentobarbital were from Sigma-Aldrich, and the DAF-FM was from Molecular Probes Invitrogen Europe BV, Poortgebouw, The Netherlands.

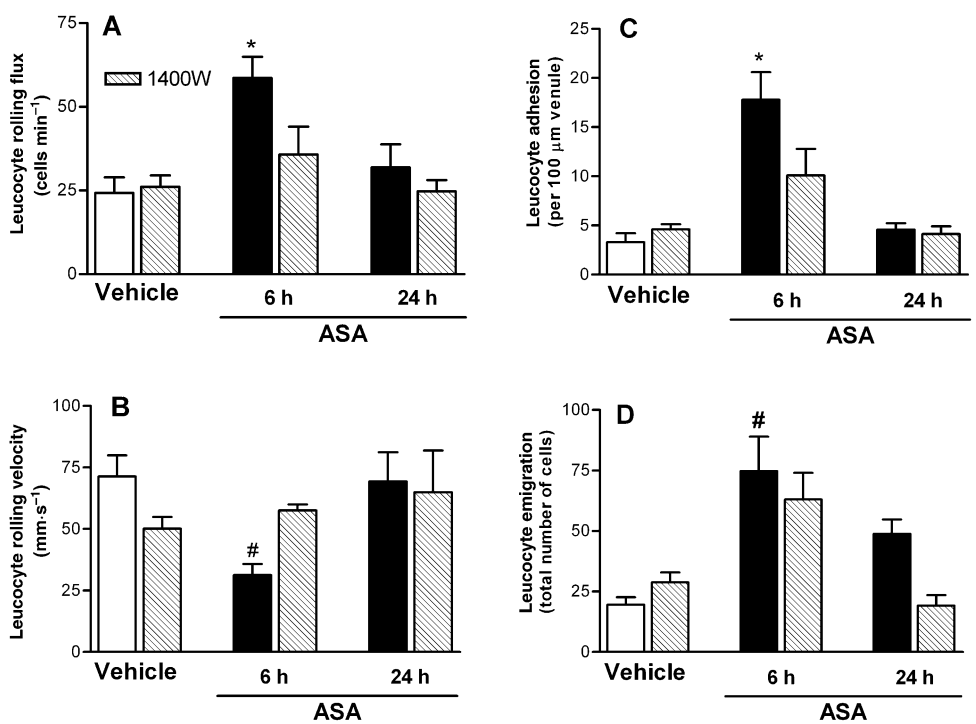

Figure 1 Effects of iNOS inhibition in the leucocyte–endothelial cell interactions induced by aspirin in rat mesentery. Aspirin induced a significant increase in leucocyte rolling flux (A), adhesion (C) and emigration (D), with a parallel reduction in rolling velocity (B), in mesenteric postcapillary venules, analysed 6 h after dosing. These interactions returned to basal values 24 h after dosing, except the number of migrated leucocytes which still remained high at this time-point. Pretreatment with an iNOS inhibitor (1400W, 5 mg·kg^{-1}, i.p.) significantly reduced the increase in leucocyte rolling and adhesion induced by aspirin at time 6 h, while it did not significantly modify the number of migrated leucocytes. However, 24 h after dosing, administration of 1400W significantly reduced the number of migrated leucocytes compared with animals receiving aspirin alone. Results are mean ± SEM. *P < 0.05 versus all experimental groups in the same graph, and #P < 0.05 versus vehicle-treated group.

Results

The role of iNOS-derived NO in aspirin-induced leucocyte–endothelial interactions

A significant increase in leucocyte rolling flux, adhesion and emigration, and a decrease in leucocyte rolling velocity were observed in the mesenteric venules of animals treated 6 h previously with aspirin, compared with vehicle-treated rats. Pretreatment with 1400W significantly reduced the increase in rolling and adhesion to the vascular endothelium, and the decrease in rolling velocity produced by aspirin, but it did not significantly modify the increase in migrated leucocytes (Figure 1).

When leucocyte–endothelial interactions were analysed 24 h after aspirin administration, levels of leucocyte rolling, rolling velocity and adhesion were similar to those reported in control rats, and the same was true for rats receiving the iNOS inhibitor before aspirin. However, the number of migrated leucocytes remained high in aspirin-treated rats, while animals receiving 1400W before aspirin presented a similar number of migrated leucocytes to that detected in control animals (Figure 1).

Venular diameters and venular wall shear rate were similar in all groups. Control rats presented a mean systemic arterial blood pressure of 118 ± 3 mm Hg, and no significant changes were induced by treatment with aspirin (117 ± 4 mm Hg), 1400W (111 ± 2 mm Hg) or the combination of 1400W + aspirin (113 ± 3 mm Hg), which indicates that the dose of 1400W used did not exert any effect on eNOS activity.

In vivo *pretreatment with aspirin increases NO synthesis in the mesentery of rats*

Under our experimental conditions, the mesenteric tissue did not show significant autofluorescence. After DAF-FM staining, mesenteric windows from vehicle-treated rats exhibited a generalized weak fluorescence signal, which did not seem to be indicative of NO synthesis as addition of L-NAME did not reduce it (Figure 2).

Mesenteric windows from aspirin-treated rats exhibited a DAF-FM fluorescent signal in cells morphologically identified as macrophages (Figure 2). Pre-incubation with L-NAME prevented the appearance of fluorescence in macrophages of the mesentery of aspirin-treated rats, confirming the fluorescence signal as being due to NO (Figure 2). In a similar manner to that observed with the *in vitro* treatment with L-NAME, animals receiving 1400W before aspirin exhibited an attenuated fluorescence in macrophages of the mesentery that was not significantly altered in samples incubated with L-NAME (Figure 2).

An intense DAF-FM fluorescent signal (30.4 ± 3.7 a.u.) was observed in cells identified as PMN leucocytes of the mesentery of control animals. This signal was significantly increased in PMN cells of the mesentery of aspirin-treated rats (40.7 ± 3.7 a.u.). In animals receiving 1400W before aspirin, DAF-FM staining in these cells was significantly attenuated (26.8 ± 4.2 a.u.) compared with animals receiving aspirin alone.

Histological analysis

The analysis of haematoxylin-stained mesenteric windows revealed the presence of macrophages and few PMN leucocytes in control animals. Aspirin induced an increase in the leucocyte infiltrate that was predominantly composed of PMN cells (Figure 3A, B). These cells were mainly located in the vicinity of the vessel and decreased gradually as the distance from the venule increased. This pattern of leucocyte infiltration was very similar at 6 and 24 h after aspirin (Figure 3C, D). Macrophages were uniformly distributed in the mesentery of control animals, and this pattern of distribution was not significantly altered by aspirin.

In aspirin-treated rats, pretreatment with 1400W did not induce any significant change in the pattern of leucocyte infiltration when observed 6 h after dosing (Figure 3A, C, E). However, 24 h after aspirin administration, rats receiving the iNOS inhibitor showed significantly less PMN and more macrophages than animals receiving aspirin alone in all areas

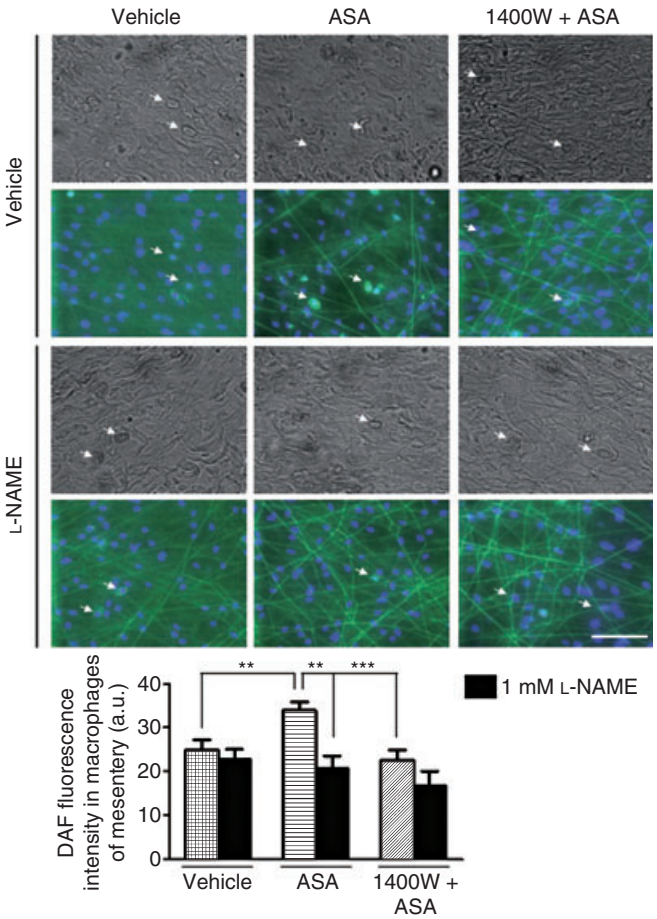

Figure 2 Fluorescence microscopy images of whole-mount preparations of mesentery. Bright field images of the mesentery showing macrophage morphology (white arrows); Hoechst 33342 staining (nuclear fluorochrome, blue) showing the nuclei of the different cells and DAF-FM staining (indicative of NO synthesis; green) in the cytoplasm of macrophages (white arrows) of vehicle or L-NAME (1 mM) pre-incubated mesentery of animals receiving the different treatments; L-NAME failed to modify DAF-FM fluorescence in the mesentery of control or aspirin (ASA) + 1400W-treated rats, while significantly reduced DAF-FM fluorescence in macrophages of aspirin-treated rats. Scale bar: 50 μm. Graph showing the quantification of DAF-FM fluorescence present in macrophages of the mesentery. Results express means ± SEM ($n \geq 3$).**$P < 0.01$ and ***$P < 0.001$.

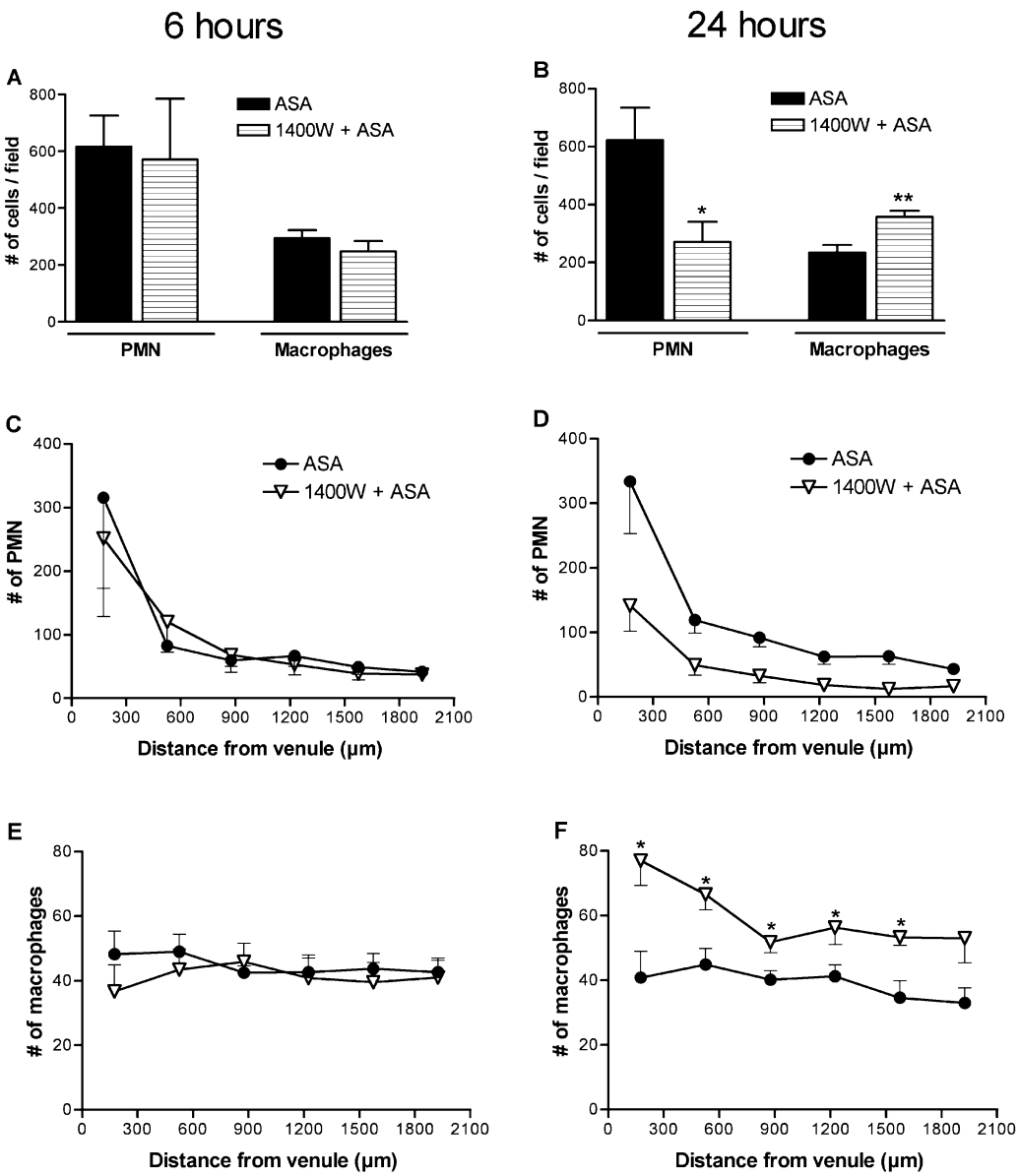

Figure 3 Effects of iNOS inhibition in the pattern of leucocyte infiltration induced by aspirin in rat mesentery. (A) and (B) show the number of PMN and macrophages in the total area analysed. Pretreatment with the iNOS inhibitor 1400W (5 mg·kg^{-1}, i.p.) did not affect the density of PMN or of macrophages at 6 h after administration of aspirin (ASA). However, rats receiving 1400W before aspirin showed significantly less PMN and more macrophages 24 h after dosing. In (C)–(F), results correspond to the number of PMN and macrophages in rectangular areas of 350 × 2100 µm parallel to the venule. Results are mean ± SEM. *$P < 0.05$ and **$P < 0.01$ versus corresponding value in the ASA group.

analysed ((Figure 3B, D, F). The increase in the number of macrophages was more pronounced in the perivascular tissue (Figure 3F). Animals receiving 1400W alone exhibited a similar pattern of cell distribution to that observed in control rats.

iNOS immunostaining

Analysis of the expression of iNOS in the mesenteric tissue of aspirin-treated rats revealed a positive signal limited to PMN and macrophages (Figure 4A). Pretreatment with 1400W did not significantly alter the pattern of iNOS expression induced by aspirin. No iNOS immunoreactivity was observed in endothelial cells of venules (Figure 4A).

HIF-1α immunostaining

Immunostaining of HIF-1α revealed the presence of this molecule in some of the PMN and macrophages present in the mesenteric tissue of control animals (Figure 4B). Aspirin administration induced a slight increase in the absolute number of HIF(+) PMN compared with control animals, but, given the net increase in the total number of PMN induced by aspirin, a similar percentage of PMN HIF(+) was observed in both groups. This percentage was not significantly different between 6 and 24 h after aspirin administration (Figure 5E). In aspirin-treated rats, iNOS inhibition did not induce any significant change in the absolute number of HIF(+) PMN (Figure 5A, B), but significantly increased the proportion of

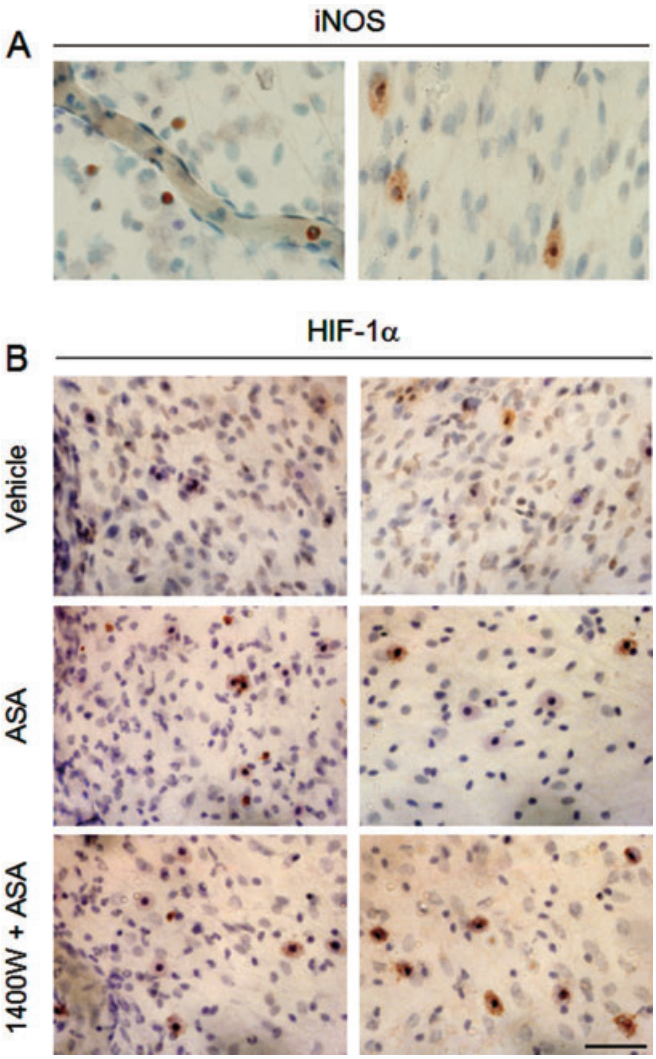

Figure 4 Inmunostaining for iNOS (A) and HIF-1α (B) in leucocytes of the mesentery of rats. (A) Positive immunostaining for iNOS was observed in PMN leucocytes and macrophages of the mesentery of rats, 6 h after treatment with aspirin. Scale bar: 20 μm. (B) Positive immunostaining for HIF-1α was observed in PMN leucocytes and macrophages of the mesentery of animals, 6 h after treatment with vehicle, aspirin or 1400W + aspirin. Scale bar: 50 μm. Representative images of the different experimental groups show an increased number of HIF-1α(+) macrophages in the mesentery of animals receiving 1400W + aspirin. In all cases, left panels show images near the vessels, and right panels show representative images in areas located distant from the vessel.

PMN expressing this transcription factor between 6 and 24 h after dosing (Figure 5E) due to the reduced number of total PMN observed at 24 h in this experimental group (Figure 3B).

Regarding macrophages, aspirin did not significantly modify the number of HIF(+) macrophages compared with control animals, either at 6 or 24 h. Administration of the iNOS inhibitor 1400W before aspirin significantly increased the number and the percentage of HIF(+) macrophages compared with animals receiving aspirin alone when observed 6 h after administration (Figure 5C, D and F). The number of HIF(+) macrophages in 1400W-treated animals remained higher 24 h after aspirin administration, but the percentage was similar to that observed in rats receiving only aspirin due to the net increase in the total number of macrophages induced by the inhibitor (Figure 3F).

In order to confirm the specific role of iNOS activity in the changes reported, we used another iNOS selective inhibitor – L-NIL (5 mg·kg^{-1}, i.p.) – and observed a similar pattern of response to that observed with 1400W. Pretreatment with L-NIL significantly ($P < 0.05$) increased the proportion of HIF(+) macrophages in the mesentery of aspirin-treated rats (56 ± 11%, $n = 4$) 6 h after dosing with respect to rats treated with aspirin alone (34 ± 3%, $n = 5$). In a similar manner, L-NIL induced a 53 ± 9% reduction ($P < 0.05$, $n = 3$) in the number of PMN in the mesentery of aspirin-treated rats 24 h after dosing.

CD36 immunostaining

Analysis of CD36 (a class B scavenger receptor) immunostaining in the mesentery of vehicle-treated animals revealed a positive signal in 54% of the cells morphologically identified as macrophages (Figure 6). The percentage of CD36(+) macrophages was significantly decreased (36 ± 1%) in the mesentery of aspirin-treated rats, while the total number of macrophages counted in the area analysed was not significantly different between the two experimental groups. Pretreatment with 1400W in animals receiving aspirin revealed a similar percentage of CD36(+) macrophages to that observed in the mesentery of vehicle-treated rats (53 ± 1%) (Figure 6).

Discussion

The present study demonstrated that inhibition of iNOS-derived NO synthesis promoted HIF-1α stabilization and increased expression of CD36 in macrophages of the mesentery of aspirin-treated rats while accelerating the clearance of leucocytes from the inflammatory site.

Experiments using intravital microscopy revealed that challenge with a gastrolesive dose of aspirin promoted the formation of an acute inflammatory reaction in the gut, which was evident in the increased leucocyte rolling and adhesion observed in the mesenteric venules of the rats 6 h after dosing. These interactions were relatively short lived, as a return to normal levels was observed 18 h later. However, the resulting inflammatory infiltrate in mesenteric tissue was quantitatively and qualitatively very similar both 6 and 24 h after dosing, and was composed of abundant PMN, as expected in an acute inflammatory reaction, and a lower number of macrophages.

We observed that pharmacological blockade of iNOS activity significantly reduced leucocyte rolling and adhesion in mesenteric venules 6 h after aspirin administration, indicating that iNOS promoted the recruitment of leucocytes in response to aspirin by favouring the interaction of leucocytes with the endothelium. This pro-inflammatory effect of iNOS-derived NO is compatible with the observed pattern of expression of this isoenzyme; we found iNOS in leucocytes, where NO could be expected to have a pro-inflammatory role (Trifilieff *et al.*, 2000; Jenei *et al.*, 2006; Krieglstein *et al.*, 2007), and not in endothelial cells, where iNOS activity seems to

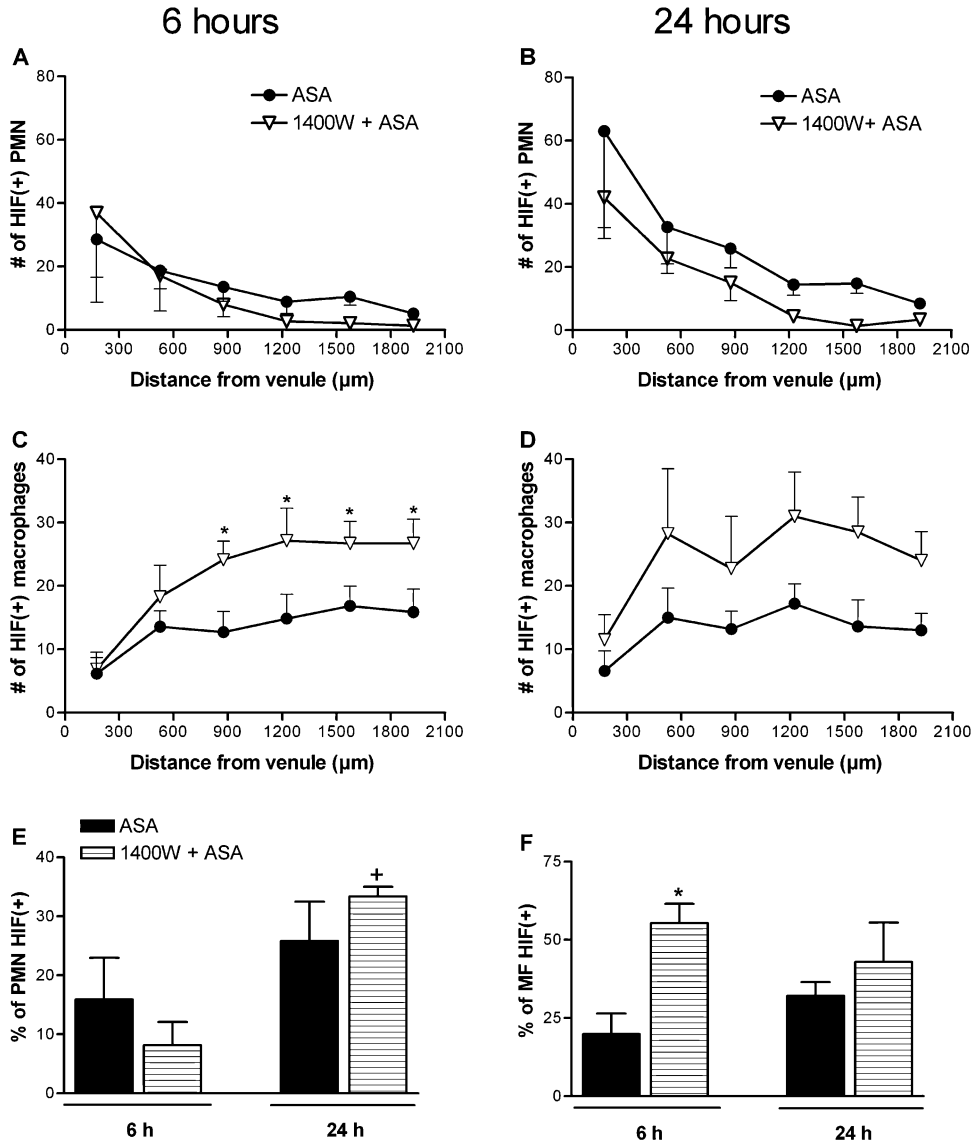

Figure 5 Effect of iNOS inhibition in the stabilization of HIF-1α in mesenteric PMN and macrophages of aspirin-treated rats. In (A)–(D), results correspond to the number of PMN or macrophages expressing HIF-1α in rectangular areas of 350 × 2100 μm parallel to the venule. Pretreatment with the iNOS inhibitor 1400W (5 mg·kg^{-1}, s.c.) did not modify the number nor the pattern of distribution of HIF-1α(+) PMN at 6 or 24 h with respect to the situation observed in aspirin (ASA)-treated rats (A, B). However, rats receiving 1400W showed an increased number of HIF-1α(+) macrophages at both time-points (C, D). (E) and (F) represent the percentage of HIF-1α(+) PMN or HIF-1α(+) macrophages in the total area analysed. No significant changes in the proportion of PMN or macrophages positive for HIF-1α were observed from 6 to 24 h in aspirin-treated rats. However, in rats receiving the iNOS inhibitor, the percentage of HIF-1α(+) PMN was significantly higher at 24 h because the total number of PMN has greatly diminished (E). The percentage of HIF-1α(+) macrophages was increased by iNOS inhibition at 6 h, while the effect of 1400W at 24 h was probably attenuated by the increased number of macrophages observed at that moment (F). Results are mean ± SEM. *$P < 0.05$ versus corresponding value in the aspirin group at 6 h. $^{+}P < 0.05$ versus corresponding value in the 1400W + aspirin group at 6 h.

emulate the inhibitory effect of eNOS on leucocyte adhesion (Hickey *et al.*, 1997). In addition, our results extend these observations and show an increased iNOS-derived NO synthesis in leucocytes of the mesentery of aspirin-treated rats, by showing an intense DAF-FM fluorescent signal that was prevented by *in vivo* pretreatment with 1400W. iNOS induction in macrophages could be due to the inflammatory process, although a direct action of aspirin on the expression of this protein on isolated macrophages has also been described (Molinuevo *et al.*, 2005).

Despite the inhibitory effect of iNOS blockade on leucocyte rolling and adhesion, 1400W did not significantly modify leucocyte infiltration, which suggests that these leucocytes that surround the vessels had extravasated during the period of time necessary for iNOS expression. It is significant, however, that most of the PMN observed in the mesentery 6 h after dosing disappeared over the following 18 h in rats receiving the iNOS inhibitor, while no changes in the infiltrated PMN were recorded during this time in rats treated only with aspirin. Considered together, these results suggest that

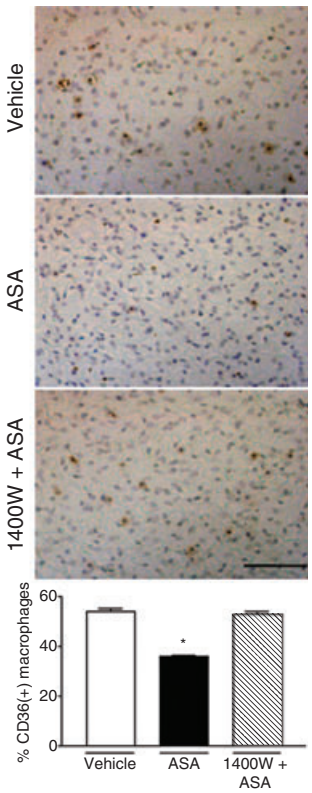

Figure 6 Effect of iNOS inhibition in CD36 immunostaining in mesenteric macrophages of aspirin-treated rats. Representative images showing positive CD36 immunostaining in macrophages of the mesentery of vehicle-treated rats. Administration of aspirin (150 mg·kg^{-1}, p.o.) induced 6 h later a reduction in CD36 immunostaining in these cells, and pretreatment with 1400W (5 mg·kg^{-1}, s.c.) significantly prevented the reduction induced by aspirin. Scale bar: 100 μm Graph shows the percentage of CD36(+) macrophages versus the total number of macrophages present in the mesenteric area analysed. Results are mean ± SEM. *$P < 0.05$ versus all groups in the graph.

iNOS-derived NO is important for the persistence of the leucocyte infiltrate. The reduction in PMN observed was accompanied by a significant increase in the number of mesenteric macrophages in rats treated with the iNOS inhibitor. Treatment with 1400W could enhance the presence of macrophages by inhibiting the pro-apoptotic effect of NO on these cells (Albina *et al.*, 1993; Liaudet *et al.*, 2000). However, it seems more likely that these macrophages come from recently extravasated monocytes. In this way, while macrophages in control and aspirin-treated rats were uniformly scattered throughout the mesenteric tissue, which is normal in resident cells, in rats pretreated with 1400W and analysed 24 h after aspirin administration, macrophages were not uniformly distributed and their number was particularly high near the vessel. This could be evidence of an acceleration in the resolution of the inflammatory process, as, in the normal course of inflammatory reactions, the initial extravasation of PMN is followed by the extravasation of monocytes which, once transformed into macrophages, eliminate the PMN and return the tissue to normality (Serhan and Savill, 2005). We cannot rule out that the higher number of PMN observed at 24 h in rats treated only with aspirin is partially due to the extravasation of new PMN after 6 h. However, if this were the case, we would expect either an increase in the granulocytic infiltrate between 6 and 24 h or, alternatively, a higher number of macrophages that would have eliminated the extra PMN.

In recent years, communication between NO and the transcription factor HIF-1, an important regulator of cellular homeostasis, has been described. In the present study, we observed HIF-1α immunostaining in macrophages of the mesentery of control animals, and a similar percentage of HIF(+) macrophages was observed in aspirin-treated rats, 6 h after dosing. Interestingly, the number of macrophages expressing HIF-1α was low near the vessel and increased gradually with increasing distance from the vessel, suggesting that hypoxia derived from the distance to vessels is involved in HIF-1α stabilization in these cells. Interestingly, iNOS inhibition with two different drugs significantly increased the percentage of HIF(+) macrophages in rats treated with aspirin, irrespective of the area analysed. These results implied that NO endogenously released in the mesentery of aspirin-treated rats prevented HIF-1α stabilization in macrophages. These findings were consistent with the role for NO in destabilizing HIF-1α induced by hypoxia in cel cultures (Mateo *et al.*, 2003). In apparent contradiction to these results, we have recently reported an increased HIF-1α immunostaining in epithelial cells of the damaged gastric mucosa, mediated by NO (Ortiz-Masia *et al.*, 2010). It is well known that the final effects of NO on HIF-1α stabilization are dependent on several factors, such as oxygen level, amount of NO available and the cellular redox state (Semenza, 2001; Erusalimsky and Moncada, 2007). These elements may vary greatly depending on local circumstances; these observations joined to differences between somatic and immune cells may account for the discrepancies observed.

The effect of iNOS inhibition on HIF-1α preceded the reduction in the extravasated PMN observed at 24 h in this group. Considering that HIF-1α expression increases in activated macrophages (Blouin *et al.*, 2004), and that a decreased bactericidal activity has been reported in macrophages from mice whose myeloid cell lineage lacks HIF-1α (Peyssonnaux *et al.*, 2005), our results endorse the hypothesis that the higher amounts of HIF-1α observed in the absence of NO enhance their phagocytic activity, and, thus, promote the removal of granulocytes. Interestingly, the iNOS inhibitor seems to affect the expression of CD36, a class B scavenger receptor whose primary function is the clearance of apoptotic cells (Febbraio *et al.*, 2001). This receptor seems to be down-regulated in the acute phases of inflammation, as occurs in the inflamed mesentery of aspirin-treated rats. Blockade of iNOS activity prevented such a decrease at 6 h, a time at which no differences in the infiltrate are observed, which suggests that NO is involved in CD36 down-regulation and that macrophages of rats treated with the iNOS inhibitor have a higher phagocytic activity. These observations are supported by studies showing a role for NO in diminishing the activity of peroxisome proliferator activated receptor-γ (Crosby *et al.*, 2006), a critical transcriptional regulator of CD36 expression. It is also interesting to point out that preliminary results from our lab show an increased up-regulation of this protein by hypoxia in macrophages (data not shown), which would suggest a correlation

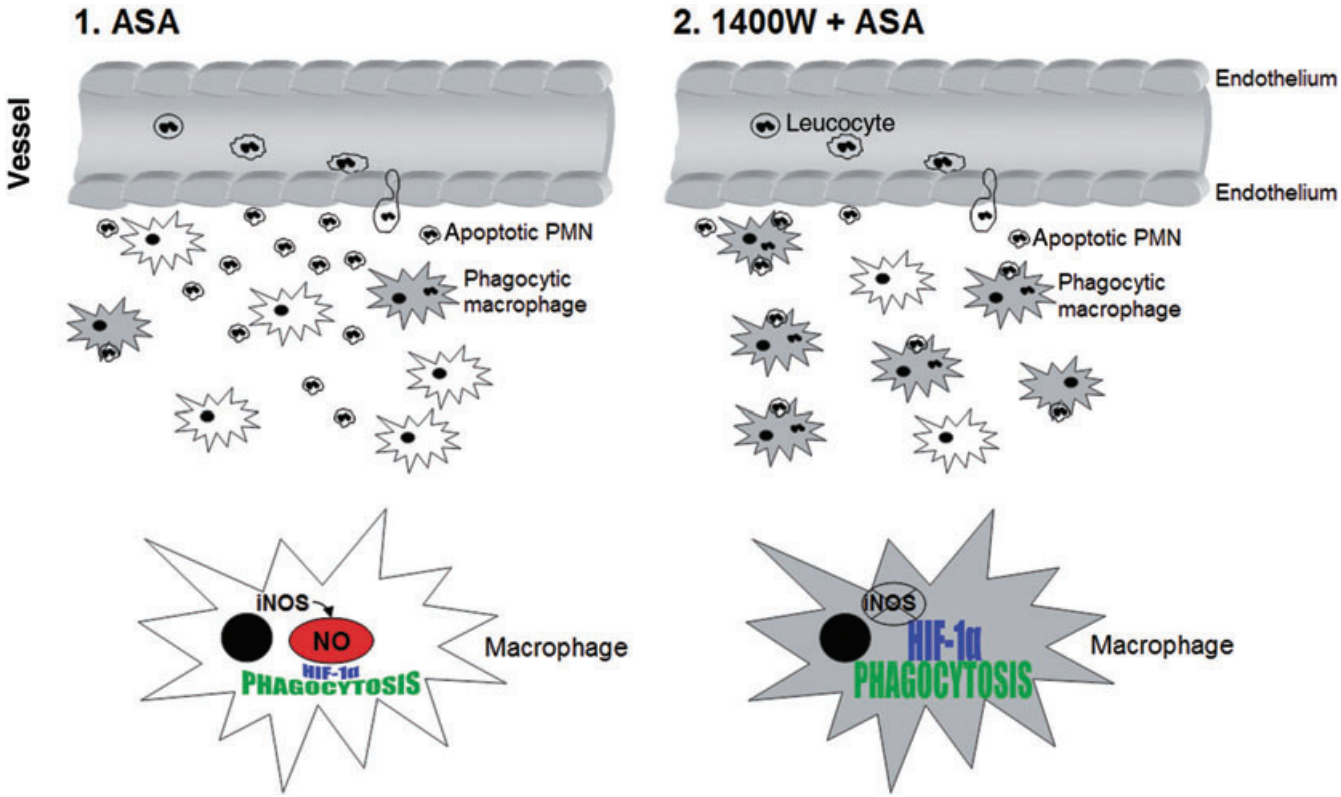

Figure 7 Diagram showing the inhibitor role of NO associated with the inflammatory process in the stability of HIF-1α in macrophages. We hypothesize that in the clear macrophages, iNOS-derived NO lowers HIF-1α levels by destabilization, and suppresses phagocytosis. Inhibition of this NO production, in the grey macrophages, leads to an increase in stabilization of HIF-1α, increased phagocytic function and clearance of the apoptotic PMN leucocytes.

between HIF-1α and CD36. Additional experiments are necessary to address this question.

HIF-1α was also detected in a small percentage of PMN. The total number of HIF-1α(+) PMN did not change significantly between 6 and 24 h after aspirin administration. A similar situation was observed in rats treated with 1400W + aspirin. However, the percentage of HIF-1α(+) PMN in these rats increased significantly between 6 and 24 h post-administration given the considerable reduction in the total number of PMN observed during this period. This implies that in rats receiving the iNOS inhibitor, the reduction in the granulocytic infiltrate is due to the disappearance of HIF-1α(–) PMN, which are less able to survive and probably enter into apoptosis (Walmsley *et al.*, 2005). We have not analysed the entrance into apoptosis of the extravasated PMN, but many studies have reported modulation of this function by NO. Further studies are needed to address this question.

In summary (Figure 7), the present study demonstrates that iNOS-dependent NO synthesis in the course of the inflammatory response induced by aspirin in the gut prevents HIF-1α stabilization in macrophages and delays resolution of the acute inflammatory process.

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Conflict of interest

None.

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