Carbon Monoxide-Releasing Molecules Modulate Leukocyte-Endothelial Interactions under Flow

Paula Urquhart, Guglielmo Rosignoli, Dianne Cooper, Roberto Motterlini, and Mauro Perretti

William Harvey Research Institute, London, United Kingdom (P.U., G.R., D.C., M.P.); and Vascular Biology Unit, Department of Surgical Research, Northwick Park Institute for Medical Research Harrow, Middlesex, United Kingdom (R.M.)

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ABSTRACT
Carbon monoxide (CO) generated by the enzyme heme oxygenase during the breakdown of heme is known to mediate a number of biological effects. Here, we investigated whether CO liberated from a water-soluble CO-releasing molecule (CO-RM) is capable of modulating leukocyte-endothelial interactions. Tricarbonylchloro(glycinato)ruthenium (II) (CORM-3), a fast CO releaser, proved to be anti-inflammatory in two distinct models of acute inflammation in vivo. In both cases, a significant reduction in neutrophil extravasation was observed. Subsequent in vitro static experiments showed that CORM-3 produced a direct effect on neutrophil (polymorphonuclear neutrophil; PMN) adhesion molecule expression; dose-dependently inhibiting platelet-activating factor stimulated CD11b up-regulation and L-selectin shedding, whereas no effect was observed on up-regulation of human umbilical vein endothelial cell (HUVEC) adhesion molecules intercellular adhesion molecule-1 or E-selectin nor on interleukin-8 chemokine production. In addition, when PMN interaction with HUVECs was studied, an inhibitory effect of CORM-3 on cell capture and rolling was observed. The effect of CORM-3 on PMN CD11b expression was mimicked by the incubation of PMN with the selective large potassium channel opener 1,3-dihydro-1-(2-hydroxy-5-(trifluoromethyl)-phenyl)-5-(trifluoromethyl)-2H-benzimidazol-2-one (NS-1619), which suggests that CORM-3 actions in this instance are mediated, at least in part, via opening of this channel. In conclusion, we have reported that CORM-3 possesses acute anti-inflammatory effects in vivo and that these are probably the result of targeting PMN activation and rolling upon the endothelium.

At the onset of an inflammatory episode, the innate immune system provides the first line of defense as polymorphonuclear neutrophil (PMN) leukocytes rapidly extravasate to the site of inflammation or infection. PMN extravasation is a tightly controlled process, which is still not fully understood; however, it is known that many contributing factors are involved not least adhesion molecule expression, cytokine and chemokine production along with a variety of other gaseous and lipid mediators (for reviews, see Louis et al., 2005; Nourshargh and Marelli-Berg, 2005). Under normal circumstances, an inflammatory response resolves itself in the fullness of time, indicating the presence of anti-inflammatory mediators that stop the extravasation of PMNs and other leukocytes. These endogenous anti-inflammatory mediators exist to counteract and regulate the proinflammatory response.

Carbon monoxide (CO) can now be regarded as one of these endogenous anti-inflammatory molecules having pleiotropic roles in a variety of physiological and pathophysiological pathways. CO is generated by the action of the ubiquitously expressed heme oxygenase (HO), an enzyme involved in heme degradation (Maines, 1997). Although the toxic effects of CO are well documented, it was only discovered in the last decade that low concentrations of CO have numerous homeostatic functions (Choi and Dolinay, 2005). The initial discovery of CO as an endogenous anti-inflammatory molecule was made in the early 1990s when platelet-activating factor (PAF) was found to be a CO donor. In addition, it was shown that CO could inhibit nitric oxide (NO) production by inhibiting inducible NO synthase (iNOS) (Maines, 1997). Since then, CO production from CO-releasing molecules has been shown to modulate leukocyte function in a number of experimental models of inflammation. CO liberated from CO-RMs can decrease neutrophil and monocyte adhesion to the endothelium.
ery of the ability of endogenous CO to cause vasorelaxation was demonstrated in isolated rabbit aortas (Furchgott and Jothianandan, 1991). Although the endothelium can actively contribute to the vasodilatory effects mediated by CO (For- esti et al., 2004), stimulation of large-conductance 
Ca
2+-
activated K
 channels (BK
Ca
) in smooth muscle cells has been identified as an additional mechanism of action. It is thought that CO acts directly on the α-subunit of the BK
Ca
, leading to increased Ca
2+ sensitivity that subsequently leads to vasorelaxation (Jaggar et al., 2002).

As a result of the sudden surge in research into CO and its properties, a novel class of compounds termed carbon monoxide-releasing molecules (CO-RMs) have been developed, and their biochemical features have been characterized (Motterlini et al., 2002). The two most recently developed are tricarbonylchloro(glycinato)ruthenium (II) (CORM-3) and sodium boranocarbonate (CORM-A1) (Fig. 1), both of which are fully water soluble and thus easy to handle, compared with previous CO-RMs, which required light activation and organic solvents (Clark et al., 2003; Motterlini et al., 2005). Upon incubation in physiological medium, both CORM-3 and CORM-A1 liberate CO gas.

A few studies using CO-RMs as a way to unveil the biological functions of CO have now been published, demonstrating the validity of these compounds to investigate the mechanisms of action of this elusive short-lived gas (Motterlini et al., 2005; Sawle et al., 2005; Stein et al., 2005; Chlopicki et al., 2006). However, little is known about the effects of CO, and CO-RMs, on endothelial-leukocyte interactions. Analysis of the rat microcirculation revealed an antiadhesive effect, via inhibition of platelet activation, of superfused CO (Morisaki et al., 2002). Therefore, we have used the new generation of water-soluble CO-RMs to study their anti-inflammatory actions, analyzing their modulation of neutrophil adhesion under flow conditions and adhesion molecule expression on human umbilical vein endothelial cells (HUVECs) and human neutrophils.

Materials and Methods

Synthesis of CORM-3 and Other Chemicals. CORM-3 was synthesized as described previously (Clark et al., 2003; Foresti et al., 2004), and it was prepared as a 10 mM stock by dissolving the compound in pure distilled water. It was kept at −20°C and defrosted before each experiment. Inactive CORM-3 (iCORM-3) was obtained by leaving CORM-3 in Dulbecco’s phosphate-buffered saline (DPBS) buffer overnight at room temperature so as to liberate all available CO from the molecule. As described in Motterlini et al. (2003), this treatment produces an inactive carrier molecule that no longer releases CO (iCORM-3), and as such it was used as a negative control to assess the direct involvement of CO in the pharmacological actions of CORM-3. As reported previously, approximately 1 mol of CO per mole of CORM-3 is liberated within 10 min after addition to DPBS (Clark et al., 2003).

\[ \text{CORM-3} \rightarrow \text{H}_2\text{O} + \text{CO} \]

Fig. 1. Chemical structures of CORM-3 and CORM-A1.
Selectin and CD11b expression was recorded as units of fluorescence where the median fluorescence intensity for 10,000 cells was measured in the FL-1 green channel (548 nm). For the anti-PSGL-1 antibody, the red FL-2 channel (590 nm) was used.

Confluent T25 flasks of HUVECs were incubated with or without TNF-α for either 4 or 24 h in the presence or absence of CORM-3/iCORM-3, and then they were plated at a density of 2 × 10⁵/well in 96-well plates and incubated with purified monoclonal antibodies: mouse anti-human ICAM-1 (0.02 mg/ml; clone 15.2; Serotec), mouse anti-human PECAM-1 (0.05 mg/ml; clone WM59; Serotec), or mouse anti-human E-selectin (0.05 mg/ml; clone 1.2B6; Serotec) for 1 h on ice before staining with fluorescein isothiocyanate-conjugated F(ab′)₂ goat anti-mouse IgG (1:100; Serotec) for a further 30 min. Isotype and unstained controls were also prepared for accurate calibration of the fluorescence-activated cell sorter machine. Flow cytometry was performed as above. ICAM-1 and PECAM-1 expression was recorded as units of fluorescence where the median fluorescence intensity for 10,000 cells was measured in the FL-1 green channel (548 nm). Supernatants from these experiments were also tested for interleukin (IL)-8 content using a selective enzyme immunoassay (548 nm). Supernatants from these experiments were also tested for interleukin (IL)-8 content using a selective enzyme immunoassay following the manufacturer’s instructions (R&D Systems, Oxford, UK).

Flow Chamber Assay. Confluent HUVEC monolayers (Cambrex Bio Science Wokingham, Ltd., Wokingham, Berkshire, UK) (up to passage 4) were stimulated with 10 ng/ml TNF-α for 4 h. Experiments with healthy volunteers were approved by the local research ethics committee. Informed consent was provided according to the Declaration of Helsinki. PMN (prepared as described above) were diluted to 1 × 10⁶/ml in DPBS supplemented with Ca²⁺ and Mg²⁺, and they were incubated with or without CORM-3 before flow for 10 min at 37°C. The flow chamber assay was run as described previously (Hayhoe et al., 2006). In brief, the chamber was placed under an Eclipse TE3000 microscope (Nikon, Melville, NY) with 40× magnification, and PMN (1 × 10⁶/ml) were perfused over the endothelial monolayers at a constant rate of 1 dye/cm² using a syringe pump (Harvard Apparatus Inc., South Natick, MA). After 8 min of perfusion, six random fields were recorded for 10 s each with a Retiga XL digital camera (Qimaging, Surrey, BC, Canada) attached to a Dell computer using StreamPix software (NorPix, Montreal, ON, Canada) to capture the images. Sequences were exported to ImagePro-Plus software (Media Cybernetics, Silver Spring, MD) for off-line analysis. Interacting PMN were manually tagged, and their movements on the endothelium were monitored. The total number of interacting cells was quantified, initially as captured cells, and then further classified as either rolling or firmly adherent (cells that remained stationary for the 10-s observation period).

Data Handling and Statistical Analysis. Flow chamber experiments were repeated at least three times. Flow cytometry was performed in duplicate or triplicate and repeated at least three times. Within each set of experiments, where applicable, different blood donors were used for each repetition. Data are reported as mean ± S.E.M., and statistical differences were determined by the Mann-Whitney U test.

Results

Anti-Inflammatory Effects of CORM-3. The acute inflammatory response to zymosan consisted of high numbers of peritoneal PMN (Fig. 2a). This effect was dramatically reduced after treatment of mice with 30 and 60 mg/kg CORM-3 compared with the vehicle, whereas the inactive preparation, iCORM-3, given at the highest dose tested, was without effect (Fig. 2a).

Challenge with met-BSA provoked a rapid inflammatory reaction studied up to the 4-h time point (Fig. 2a). Again, administration of CORM-3 provoked significant dose-dependent attenuation of the swelling, whereas iCORM-3 was inactive (Fig. 2b). Therefore, CORM-3 inhibited innate and adaptive immune responses in two distinct, yet acute, models of inflammation, as well as in two different anatomical sites.

Effect of CORM-3 on PMN Adhesion Molecule Expression. To gain information of the potential cell target(s) for the observed effects, in vitro experiments were conducted, moving to human cells to augment the translational impact of these findings. PMN incubation with PAF provoked the expected up-regulation of CD11b, and this cellular response was inhibited by CORM-3 in a concentration-dependent manner, but not by iCORM-3 (Fig. 3a). Of interest, CORM-3 also provoked a reduction in CD11b levels in control cells, perhaps indicating the occurrence of mild activation during the incubation phase. This effect of CORM-3 was not limited to PAF-stimulated PMN, because it also occurred when the N-formyl-L-methionyl-L-leucyl-L-phenylalanine peptide was used as stimulus (for example, at 100 μM CORM-3, an inhibition of 71 ± 19.9% was calculated). CORM-A1 elicited similar inhibitory responses, although higher concentrations were required to attain similar levels of inhibition as CORM-3: 61 ± 11% inhibition was calculated for 500 μM CORM-A1 (n = 3; P < 0.05 versus PAF alone).
Effect of CORM-3 on HUVEC Markers of Activation. We then tested whether CO-releasing molecules could affect HUVEC activation. To this end, TNF-α-stimulated HUVECs were tested for adhesion molecule expression (e.g., ICAM-1, PECAM-1, and E-selectin) and release of IL-8. Table 1 reports these data indicating that cell activation with the cytokine produced ICAM-1 and E-selectin up-regulation, as tested at 4- and 24-h time points, associated with modest PECAM-1 shedding. In addition, IL-8 concentrations in the supernatants were also augmented compared with untreated HUVECs. Incubation with CORM-3 was without effect on either adhesion molecule expression or IL-8 production (Table 1).

Effect of CORM-3 on PMN-HUVEC Interactions under Flow. This assay was performed because of its pathophysiological validity; cell behavior under flow conditions is certainly more comparable with in vivo conditions during the early phase of the acute inflammatory response than under static conditions. PMN incubation with CORM-3 produced a concentration-dependent reduction in the extent of cell capture to the endothelial cells (Fig. 4a). Further analysis showed that this reduction was predominantly due to an inhibition in PMN rolling (up to 50–60%; n = 4; P < 0.5; Fig. 4b) rather than PMN adhesion (Fig. 4c). Of interest CORM-3, under these conditions was optimally active at 1 μM, whereas iCORM-3 was inactive even when tested at the concentration of 10 μM.

Indications of the Possible Molecular Targets for CORM-3. In an attempt to elucidate the mechanism of action behind the inhibitory effect of CORM-3 on CD11b up-regulation, an investigation was carried out into the role of BKCa-PMN treatment with NS-1619, a selective BKCa opener, caused a significant concentration-dependent decrease in CD11b up-regulation in response to cell activation with PAF (Fig. 5a). This effect was similar to that observed with CORM-3 treatment. However, addition of paxilline (selective BKCa blocker) into the experimental system did not significantly modify PAF-induced CD11b expression (Fig. 5a).

The same "static" system was used monitoring L-selectin shedding. This time, neither NS-1619 nor paxilline affected PAF-stimulated L-selectin shedding (Fig. 5b). The same result was obtained when modulation of sGC was investigated by cell incubation with the activator YC-1 or the inhibitor ODQ (Fig. 5c).

Discussion

We describe here the acute anti-inflammatory properties of CO-RMs, and we indicate that the activated PMN, instead of...
the endothelial cell, would be the main target behind these effects. In addition, we demonstrate the ability of CORM-3 to affect PMN interaction with HUVECs under flow.

CORM-3 has been shown to reduce inflammatory cell activation in vitro (Sawle et al., 2005) and in vivo in an acute model of myocardial infarction (Guo et al., 2004). We questioned whether this compound could affect cell trafficking; hence, we tested it in the zymosan-induced peritonitis mouse model. A dose-dependent inhibition, genuinely due to the CO-releasing ability, because iCORM-3 was ineffective, was observed. These data reinforce the anti-inflammatory role of CO, and they link well with the inhibitory action of HO-1 in models of acute inflammation (Guo et al., 2004; Lam et al., 2005; Shen et al., 2005). In addition, CORM-3 properties were not tissue- and stimulus-specific in view of its efficacy on the met-BSA-induced paw swelling model. Therefore, CO has broad-ranging anti-inflammatory properties that can be successfully capitalized with these novel compounds. Both in vivo models could have been run for longer time points; however, we stopped at early points to avoid potential confounding factors, including pharmacokinetics. In addition, longer time points of the met-BSA model are likely to rely on adaptive immune multicellular components (Moore et al., 1999), thereby complicating the scenario further. Results from these two models indicate that the innate immune system can be actively inhibited by CORM-3, successfully trapping the beneficial properties of CO. This conclusion is in line with that drawn for nitric oxide-donating compounds, also shown to retain potent anti-inflammatory properties (Wallace and Cirino, 1994), as well as for the more recently described hydrogen sulfide-donating drugs (Zanardo et al., 2006).

Our study went on with the aim of identifying the cellular targets of CO: to do this, we initially conducted single-cell culture experiments under static conditions. CORM-3 produced marked and significant changes in PAF-stimulated PMN adhesion molecule expression: up-regulation of CD11b was inhibited as was L-selectin shedding. It is noteworthy that the observed inhibition of CD11b up-regulation was not specific to PAF, since similar dose-dependent inhibitions of CORM-3 were observed on N-formyl-t-methionyl-t-leucyl-t-

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**Fig. 4.** CORM-3 reduces PMN-HUVEC interactions under flow. Freshly isolated PMN (1 × 10⁶/ml) were incubated with vehicle (Ctrl), CORM-3 (0.1–10 μM), or iCORM-3 (10 μM) for 10 min at 37°C before flow over confluent HUVEC monolayers (stimulated with 10 ng/ml TNF-α for 4 h) at a constant rate of 1 dyne/cm² for an 8-min recording period. Extent of captured PMN (a), rolling PMN (b), and adherent PMN (c) as determined in six distinct fields. Absolute values for captured PMN (taken as 100%) ranged between 80 and 120 cells. Data are expressed as percentage and are mean ± S.E.M. of three distinct experiments performed with different PMN preparations and HUVEC batches (*, P < 0.05 versus Ctrl).

**Fig. 5.** Effect of BKCa and sGC modulation on PAF-induced PMN activation. a, freshly isolated human PMN (1 × 10⁶/ml) were incubated for 10 min with either the BKCa opener NS-1619 (1–20 μM) or the BKCa blocker paxilline (pax; 1–100 μM) before the addition of 1 nM PAF for further 30 min. b, human PMN (1 × 10⁶/ml) were incubated with either the sGC activator YC-1 (5–50 μM) or the sGC inhibitor ODQ (1–100 μM) before the addition of 1 nM PAF for a further 30 min (c). In either case, CD11b and L-selectin levels were measured by flow cytometry. Data are expressed as percentage and are mean ± S.E.M. of six distinct PMN preparations (*, P < 0.05 versus PAF alone; dose 0).
phenylalanine-induced CD11b up-regulation. In addition, a reduction in cell viability was not responsible for the observed inhibition, because an Alamar Blue viability study was carried out and showed no reduction in PMN metabolic activity (unpublished observations). It is possible that inhibition of CD11b up-regulation and function could account, at least in part, for the antimigratory effects displayed by CORM-3 in the peritonitis model (Perretti et al., 1993).

CORM-3 effects on PMN CD11b up-regulation, hence on cell activation, were replicated with respect to L-selectin shedding. The latter response is very sensitive, with rapid activation of membrane sheddase even after simple mishandling of the cells (Borregaard et al., 1994; Jagels et al., 1995).

Collectively, data produced with two markers of cell activation indicate that the PMN could be targeted by CORM-3, and CORM-A1 (although only in part analyzed here, i.e., on CD11b up-regulation). This is at variance with that found on HUVECs. Both membrane adhesion molecules and soluble mediator release were tested, using the well validated ICAM-1 marker and IL-8, respectively. After TNF-α stimulation, HUVEC ICAM-1 is induced by de novo protein synthesis through nuclear factor-κB activation and translocation to the nucleus (Read et al., 1995), and, often, this process is associated with PECAM-1 shedding (Wheller and Perretti, 1997), although the latter phenomenon is quite marginal (i.e., no more than 10–15% of PECAM-1 is shed; Perretti et al., 1996). CORM-3 was inactive on this response of the activated HUVECs. This is apparently in contrast to a recent report, quoted in Mannioni et al. (2006), stating the ability of this CO-RM to reduce ICAM-1 increase after PMN adhesion to the endothelial monolayers, as determined in static conditions. However, based on what was discussed above, it is very likely that the latter result is consequence of a primary inhibitory effect of the compound on the PMN. Congruently, PMN adhesion to HUVECs causes endothelial dysfunction, possibly via release of oxidative species, with delayed up-regulation of ICAM-1 on the endothelial cell (Wang and Doerschuk, 2000, 2001).

A different approach was taken by Soares et al. (2004) who described how HO-1 overexpression in HUVECs caused inhibition of TNF-α-stimulated E-selectin, ICAM-1, and VCAM expression; however, these effects were due to bilirubin production or free iron depletion and not CO production (Soares et al., 2004). The lack of effect of CORM-3 on HUVEC activation, as tested in our experimental conditions, was confirmed by determination of IL-8 release as well as prostaglandin E2 generation (unpublished observations).

Having established that the PMN, and not the HUVEC, was the likely target for CORM-3, we then tested whether the CO-releasing compound could affect PMN interaction with the HUVEC under flow. This protocol is clearly more relevant to an inflammatory condition, and it is often associated with higher cell sensitivity to treatment, probably because of the alteration induced by shear on cell membrane fluidity, and overall promptness of responsiveness. In this experimental setting, CORM-3 produced a marked attenuation of PMN capturing and rolling, but not adhesion, with an optimal concentration of 10 μM, below the maximally effective concentration determined in the single-cell static assays. Thus, this first report of CORM-3 on the interaction of human PMN and HUVECs under flow conditions is strongly indicative of a cellular mechanism rapidly translatable to early phases of the inflammatory reaction. In addition, iCORM-3 was ineffective. Collectively, these cellular studies indicate that the actions of CORM-3 upon the PMN would affect the cellular responsiveness after flow on an activated endothelium. Since we have recently reported that L-selectin is partly responsible for PMN rolling on HUVEC monolayers, as is PSGL-1 (Hayhoe et al., 2006), our expectation would have been no-effect on L-selectin shedding. However, it is clear that the two systems, static versus flow, cannot be directly compared, and that PMN activation, hence L-selectin shedding, in the test tube was simply used to assess sensitivity of this cell type to CORM-3 inhibition. It is likely that other distinct molecular responses of the PMN could underlie CORM-3 efficacy in the flow chamber assay; the valence of this in vitro observation on the inflammatory response remains high.

BKca and sGC have both been indicated as possible targets for CO (Jaggar et al., 2002; Foresti et al., 2004). So, in the final part of the study, we investigated whether blocking these two putative targets would affect CORM-3 effects, and the single-cell culture with CD11b up-regulation as readout was chosen for its simplicity. PMN incubation with a selective BKca opener, NS-1619, produced a significant concentration-dependent reduction in PAF-induced CD11b up-regulation, with maximal efficacy at 20 μM. However, NS1619 did not exert any protective effects on L-selectin shedding; therefore, although a CORM-3 effect on BKca could be advocated for inhibition of CD11b up-regulation, distinct mechanisms must underlie inhibition of L-selectin shedding. Interestingly, when we combined CORM-3 and NS1619 there was no additive effect on CD11b up-regulation (unpublished observations). We propose that the effect of CORM-3 effect upon CD11b is, at least in part, via the activation of BKca; hence, the two compounds were mutually exclusive.

The BKca blocker paxilline was inactive, possibly because the channel is not endogenously activated during the cellular response to PAF. Alternatively, it is possible that PAF-induced calcium concentration could have competed with paxilline for binding to the BKca, because the cation and the blocker can occupy the channel simultaneously (Sanchez and McManus, 1996). We also addressed the involvement of sGC in regulation by CO-RM of L-selectin shedding, but we found that neither YC-1 nor ODQ influenced human PMN L-selectin shedding. This is in line with a recent report showing that the inhibitory effect of CORM-3 on human platelet aggregation is a cGMP-independent mechanism (Chlopicki et al., 2006). Further investigations into events affected by CORM-3 to inhibit L-selectin shedding are definitely warranted.

How does the level of CO released from CORM-3 compare with physiological yields of CO derived from heme oxygenase? The basal production of CO derived from the degradation of heme by heme oxygenase in mammals is estimated to be 1 to 6 μmol/kg/h (Berk et al., 1974); however, the amount of CO produced once HO-1 has been induced to counteract a stress condition has never been determined, and the turnover of the heme pool for each specific cell type is unknown. Therefore, the amount of CO being produced will depend on the degree of HO-1 induction in a given cell and on the amount of “free heme” available to be used as substrate. Moreover, the spatial and temporal distributions of CO and the HO isoforms from which it is derived make the action of
endogenously generated CO difficult to replicate even if we knew the concentration(s) of CO-RMs that would mimic physiological or “supraphysiological” amounts of CO. This is consistent with the idea that the protective role of the HO-1/CO pathway is a function not only of how much CO is produced but also where it is produced and “how the target(s) is responsive to its signal”. Despite these limitations, the use of 100 μM CORM-3 seems to be within the physiological or supraphysiological concentrations if one considers that in normal nonsmoking subjects the concentration of carboxyhemoglobin is 1 to 2%, equivalent to 98 to 196 μM CO (of note, the concentration of heme from hemoglobin in man is in the range of 8–10 mM).

In conclusion, we have reported here that CORM-3 possesses acute anti-inflammatory effects in vivo and that these are the result of targeting PMN activation and rolling upon the endothelium. A potential involvement of the BKCa+ and not sGC, is tentatively proposed, although more specific studies are needed to clarify this aspect and to identify the molecular targets for CO released from CORM-3 in PMN. Therefore, an effective way to exploit the protective actions ascribed to low CO concentrations is certainly with CORM-3 and similar compounds, which could be developed as novel anti-inflammatory therapeutics.

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Address correspondence to: Dr. Mauro Perretti, William Harvey Research Institute, Barts and The London, Charterhouse Square, London EC1M 6BQ, UK. E-mail: m.perretti@qmul.ac.uk