Endogenous generation and protective effects of nitro-fatty acids in a murine model of focal cardiac ischaemia and reperfusion

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Aims	Nitrated fatty acids (NO_2-FA) have been identified as endogenous anti-inflammatory signalling mediators generated by oxidative inflammatory reactions. Herein the *in vivo* generation of nitro-oleic acid (OA-NO_2) and nitro-linoleic acid (LNO_2) was measured in a murine model of myocardial ischaemia and reperfusion (I/R) and the effect of exogenous administration of OA-NO_2 on I/R injury was evaluated.
Methods and results	In C57/BL6 mice subjected to 30 min of coronary artery ligation, endogenous OA-NO_2 and LNO_2 formation was observed after 30 min of reperfusion, whereas no NO_2-FA were detected in sham-operated mice and mice with myocardial infarction without reperfusion. Exogenous administration of 20 nmol/g body weight OA-NO_2 during the ischaemic episode induced profound protection against I/R injury with a 46% reduction in infarct size (normalized to area at risk) and a marked preservation of left ventricular function as assessed by transthoracic echocardiography, compared with vehicle-treated mice. Administration of OA-NO_2 inhibited activation of the p65 subunit of nuclear factor κB (NFκB) in I/R tissue. Experiments using the NFκB inhibitor pyrrolidinedithiocarbamate also support that protection lent by OA-NO_2 was in part mediated by inhibition of NFκB. OA-NO_2 inhibition of NFκB activation was accompanied by suppression of downstream intercellular adhesion molecule 1 and monocyte chemotactic protein 1 expression, neutrophil infiltration, and myocyte apoptosis.
Conclusion	This study reveals the *de novo* generation of fatty acid nitration products *in vivo* and reveals the anti-inflammatory and potential therapeutic actions of OA-NO_2 in myocardial I/R injury.
Keywords	Ischaemia/reperfusion • Nitric oxide • Nitro-fatty acids • Reactive oxygen species • Oxides of nitrogen • Lipid signalling

1. Introduction

Reperfusion injury is a major contributor to tissue damage during myocardial infarction, accounting for up to 50% of final infarct volume.[1] Despite considerable insight into the mechanisms of cardiac ischaemia and reperfusion (I/R) injury, no effective therapeutic strategies have been introduced into clinical practice.

Extensive experimental evidence reveals a role for reactive oxygen species (ROS) in mediating myocardial reperfusion injury.[2] This concept was first introduced in 1973 as the 'oxygen paradox',[3] based on the observation of reoxygenation-induced tissue injury in hypoxic rat hearts and was reinforced by interventional animal studies using antioxidant-based therapeutic strategies, ROS-generating systems,[4–6] and finally, by direct measurement of

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ROS generation *in vivo*.[7] Various sources of increased ROS generation during myocardial reperfusion yield products that readily undergo secondary reactions with radical and non-radical species, including nitric oxide (•NO) and its metabolites.[8,9] This oxidative inflammatory milieu generated during reperfusion injury consists of a broad spectrum of oxidizing, nitrosating, and nitrating species and their products.[10]

Nitrated unsaturated fatty acids (NO$_2$-FA) are endogenously occurring products of oxidant-induced nitration reactions. NO$_2$-FA display predominantly anti-inflammatory signalling actions, which to date have only been exemplified in biochemical and cellular models of inflammation.[11] Recently, potential cardioprotective actions of NO$_2$-FA were suggested from studies of cultured cardiomyocytes subjected to simulated I/R injury, with these protective effects ascribed to mild mitochondrial uncoupling.[12] In this study, nitration products of linoleic acid were endogenously generated during ischaemic preconditioning (IPC) of Langendorff-perfused rat hearts, suggesting a possible contribution of NO$_2$-FA to the protection lent by IPC.

Herein, we translate these *in vitro*-based observations to an *in vivo* model of cardiovascular injury by first providing evidence for the *in vivo* formation of NO$_2$-FA in a murine model of focal myocardial I/R. We then show that administration of nitro-oleic acid (OA-NO$_2$) during ischaemia, at clinically relevant times, markedly reduces myocardial infarct size and preserves left ventricular function. These protective effects are in part mediated by inhibition of nuclear factor κB (NFκB) activation via post-translational modification of the p65 subunit by electrophilic NO$_2$-FA, resulting in local and systemic down-regulation of downstream pro-inflammatory signalling. These studies reveal that tissue supplementation with an endogenous by-product of inflammatory reactions serves to inhibit myocardial I/R injury.

2. Methods

2.1 Animal studies

Male, 8- to 12-week-old C57/BL6 wild-type mice (Jackson Laboratory, Bar Harbor, ME, USA) were used for all studies. The investigation conforms with 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) and was approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh (protocol number: 0710454).

2.2 I/R protocol

Mice were anaesthetized by intraperitoneal injection of 0.5 mg/g body weight of avertin (2-2-2-tribromoethanol, Sigma-Aldrich, USA). After lateral thoracotomy of the fourth intercostal space, a suture was placed around the left coronary artery after retraction of the left atrial appendage and the artery was ligated with a bow tie. After 30 min of ischaemia, the bow tie was released, the suture left in place for determination of infarct size (see below), and the chest cavity was closed; and 20 nmol/g bodyweight OA-NO$_2$ in polyethylenglycol/ethanol (85:15, vol/vol) or 100 μL of vehicle (polyethylenglycol/ethanol, 85:15) were injected 15 min prior to reperfusion. Two further groups of animals were treated with OA-NO$_2$ via Alzet osmotic mini-pumps over a period of 3 days prior to induction of ischaemia and intraperitoneal injection of 20 nmol/g body weight OA-NO$_2$ at the time of reperfusion, respectively. A 6 mg/kg dose was delivered via a 7-day osmotic mini-pump. In some cases, the NFκB-inhibitor pyrrolidi-nedithiocarbamate (PDTC, 150 mg/kg, Sigma-Aldrich) was administered 30 min prior to surgery. OA-NO$_2$ was synthesized as previously.[13,14] Sham surgery animals underwent removal of the pericardium. Infarct size was determined using Evan's blue dye. Echocardiographic studies were performed before and 24 h after induction of myocardial ischaemia (see *Scheme 1* for an illustration of the experimental protocol).

2.3 Detection and quantitative analysis of NO$_2$-FA

For detection of endogenous NO$_2$-FA levels, mice underwent surgery as described above. To assess the impact of duration of ischaemia on the formation of NO$_2$-FA, ischaemic periods were varied, with times ranging from 15 min to 1 h. In one group, 20 nmol/g body weight OA-NO$_2$ was administered 15 min prior to reperfusion. In addition to sham-operated mice, another group consisted of mice undergoing ligation of the left coronary artery without subsequent reperfusion. In these groups, reperfusion times were 30 min and then heart tissue was removed, lipid extraction was performed, and OA-NO$_2$ and nitro-linoleic acid (LNO$_2$) assessment was carried out by liquid chromatography/mass spectrometry (LC/MS) as before.[14]

2.4 Activation of NFκB p65 subunit

Hearts were harvested 1 h after reperfusion, homogenized and nuclear extraction was performed according to manufacturer recommendations using a commercially available nuclear extract kit (Active
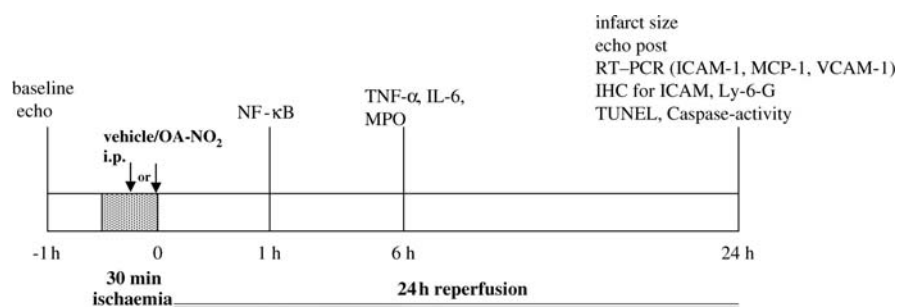
Scheme 1 Experimental protocol for the assessment of OA-NO$_2$ effects in the murine I/R model.

Motif, Carlsbad, CA, USA). Activation of the NFκB p65 subunit was detected and quantified via ELISA analysis (TransAM p65 chemikit, Active Motif).

2.5 Immunoprecipitation and assessment of p65 nitroalkylation

For immunoprecipitation of p65, homogenates from hearts harvested after 1 h of reperfusion were incubated with a rabbit polyclonal NFκB p65 antibody (abcam, 1:160) for 1 h at 4°C. Thereafter, the mixture was incubated with agarose (Protein A/G PLUS-Agarose, Santa Cruz Biotechnology, USA, 1:50). To control for non-specific binding of $OA-NO_2$ to agarose beads, the NFκB p65 antibody was omitted in control samples. To release $OA-NO_2$ covalently bound to p65, immunoprecipitates were incubated with 500 mM of β-mercaptoethanol (BME) for 2 h at 37°C. This exchange reaction reveals the reversible adduction of proteins by electrophiles in biological samples via the exchange reaction of protein-adducted species (e.g. $OA-NO_2$) to BME and subsequent LC/MS analysis of BME-electrophile adduct formation.[13,14] After BME incubation, samples were centrifuged at 1000 g, for 10 min at 4°C and were analyzed by LC/MS as above.

2.6 Tumour necrosis factor α and interleukin 6 serum levels

Blood was drawn from the inferior vena cava after 6 h of reperfusion and directly transferred into serum separator tubes (BD Microtainer, USA). After 2 h samples were centrifuged at room temperature at 6000 g for 10 min. Serum levels of tumour necrosis factor α (TNFα) and interleukin 6 (IL-6) were determined by ELISA according to manufacturer recommendations (Quantikine, R&D Systems, USA).

2.7 Myeloperoxidase tissue levels

For determination of tissue myeloperoxidase (MPO) levels, hearts were harvested 24 h after reperfusion and the left ventricle directly homogenized in lysis buffer [200 mM NaCl, 5 mM EDTA, 10 mM Tris, 10% glycerol, complete EDTA-free protease inhibitor cocktail (Roche Diagnostics, USA)]. Protein concentration was determined by BCA assay. MPO levels were assessed by ELISA (Hycult Biotechnology bv, The Netherlands).

2.8 Real-time quantitative polymerase chain reaction

Hearts were harvested after 24 h and RNA was isolated from the left ventricular free wall and quantitative mRNA expression assessed by real-time PCR using TaqMan gene expression assays for intercellular adhesion molecule 1 (ICAM-1, NM010493.2), monocyte chemotactic protein 1 (MCP-1, NM_011333), and vascular cell adhesion molecule (VCAM-1, NM_011693.2, Applied Biosystems, USA). All values were normalized to $β_2$-microglobulin (NM_009735.3).

2.9 Immunofluorescence analysis of ICAM-1 and Ly-6-G

Six micrometre cross-sections were incubated for 1 h with a rat polyclonal antibody to ICAM-1 (abcam, USA, 1:25) and a rabbit polyclonal antibody to CD31 (abcam, 1:20) or a rat polyclonal antibody to Ly-6-G, specific to neutrophils (abcam, 1:20).

2.10 Assessment of apoptosis

End labelling of exposed DNA-fragments was performed with a TUNEL in situ cell death detection kit (Roche Diagnostics, Switzerland) as suggested by the manufacturer. In 10 random fields per slide, all nuclei and all TUNEL-positive nuclei were automatically counted using MetaMorph and the percentage of TUNEL-positive nuclei was calculated.

Caspase 3 activity was assessed in homogenized heart tissues harvested after 24 h of reperfusion according to manufacturer recommendations (BD Pharmingen™, BD Biosciences, USA).

2.11 Statistical analysis

Results are expressed as mean ± standard deviation. Continuous variables were tested for normal distribution using the Kolmogorov–Smirnov test. Statistical analysis was performed using one-way ANOVA followed by Bonferroni post hoc test or unpaired Student's t-test as appropriate. A value of $P < 0.05$ was considered statistically significant. All calculations were carried out using SPSS version 15.0.

3. Results

3.1 Endogenous formation of NO_2-FA

Analysis of myocardial tissue from sham-operated and mice following 30 min of ischaemia and 30 min of reperfusion revealed the generation of $OA-NO_2$ in a concentration of 9.5 ± 4.7 nmol/L ($n = 3$) after I/R, with no detectable $OA-NO_2$ in sham-operated animals and mice with myocardial infarction without reperfusion (Figure 1A). The HPLC-MS identification of $OA-NO_2$ was based on (i) identical chromatographic retention times when comparing endogenously formed $OA-NO_2$ with a synthetic $[^{13}C]-OA-NO_2$ internal standard (Figure 1B–D), (ii) similar ratios of ion intensity for synthetic, endogenously produced and ^{13}C-labelled $OA-NO_2$ when comparing the two main products of $OA-NO_2$ collision induced dissociation m/z 326/46 and m/z 326/279 (Figure 1E), and (iii) the manifestation of an electrophilic reactivity via the formation of an adduct with BME (see Supplementary material online, Figure S1).[13,14] In mice receiving intraperitoneal injection of synthetic $OA-NO_2$ 15 min prior to reperfusion, the myocardial tissue concentration of $OA-NO_2$ after 30 min of reperfusion was 230 ± 4 nmol/L.

Following reperfusion a nitro-fatty acid with the mass transitions of LNO_2 was formed that co-eluted with an LNO_2 standard synthesized from trans- rather than cis-linoleic acid (Figure 2A–D). These data reveal that trans-LNO_2 generation was favoured under these conditions, with a calculated concentration of 17.3 ± 4.7 nM. The specific position of the trans double bond and the location of the vinyl NO_2-group remains to be determined. Identification of this product as the electrophilic nitroalkene derivative of LNO_2 was affirmed by LC/MS analysis of the characteristic fragmentation pattern upon collision-induced dissociation, co-elution with a synthetic trans-LNO_2 standard, and reactivity with BME. In addition to $OA-NO_2$ and trans-LNO_2 derivatives, additional hydroxyl and keto derivatives of LNO_2 were detected by LC/MS analysis that also displayed electrophilic reactivity (Figure 3A–D). Variation of the ischaemic period from 30 to 15 min and 1 h, respectively, did not result in changes in tissue levels of oleic and linoleic acid nitration products (data not shown).
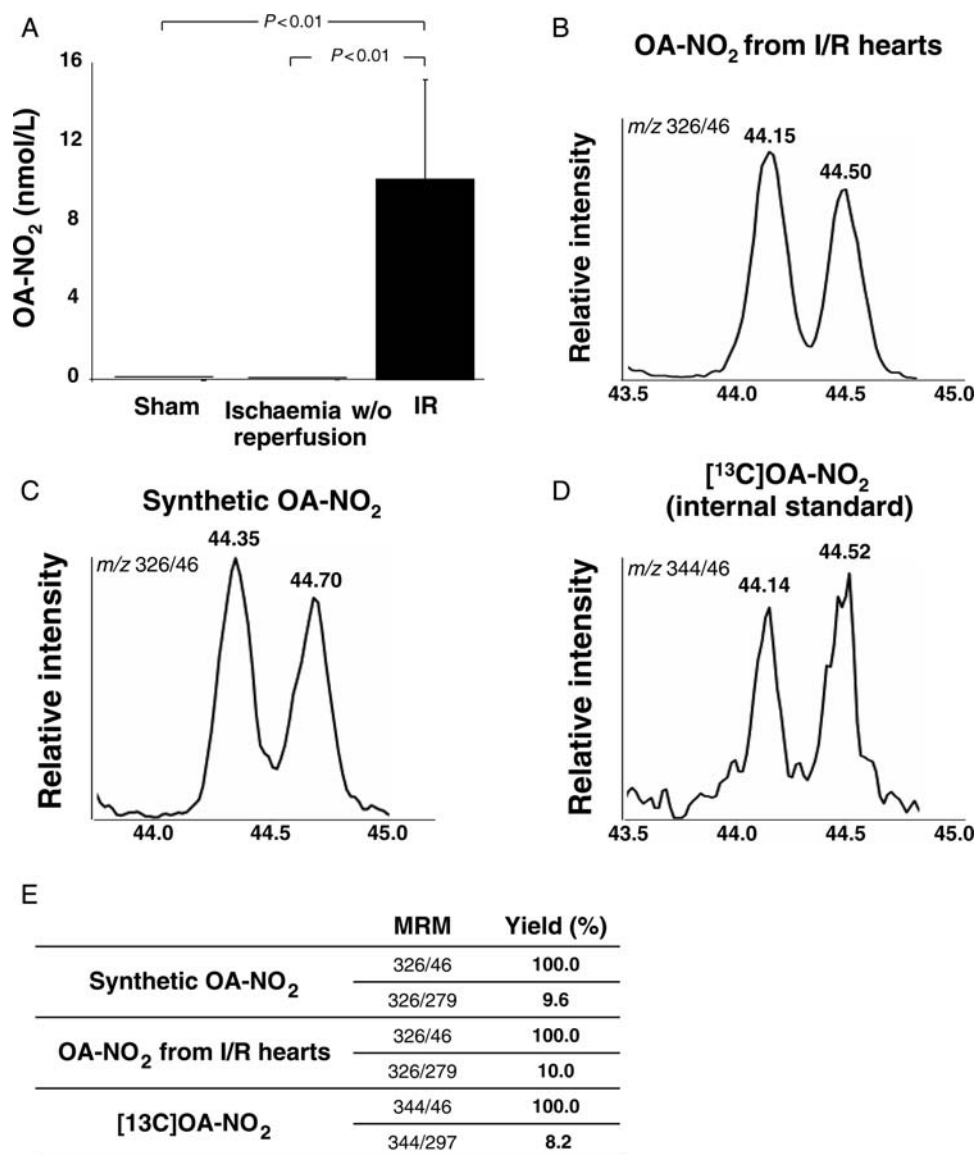
Figure 1 Endogenous generation of OA-NO$_2$ following ischaemia and reperfusion. (*A*) Tissue concentration of OA-NO$_2$ in hearts from mice undergoing 30 min of ischaemia and 30 min of reperfusion, ischaemia without reperfusion or sham surgery. (*B–D*) Characterization of OA-NO$_2$ in heart tissue from mice subjected to I/R injury. The chromatograms show co-elution of OA-NO$_2$ extracted from heart tissue with synthetic OA-NO$_2$ (*C*) and the internal standard (*D*). (*E*) The table shows the peak area ratios corresponding to the two main products of OA-NO$_2$ collision induced dissociation: *m/z* 326/279 (neutral loss of HNO$_2$) and *m/z* 326/46 (formation of NO$_2^-$), further confirming the identity of OA-NO$_2$ extracted from the heart tissue.

3.2 Nitro-oleate-induced reduction in myocardial infarct size

Following 30 min of myocardial ischaemia and 24 h of *in vivo* reperfusion, mice treated with OA-NO$_2$ 15 min prior to reperfusion exhibited a significant reduction of the IA/AAR (27.6 ± 7.9 vs. 50.1 ± 8.7%, $P < 0.01$) and IA/LV ratio (14.4 ± 3.9 vs. 25.8 ± 7.7%, $P < 0.01$) when compared with vehicle-treated animals (*Figure 4A–C*). Administration of OA-NO$_2$ 3 days prior to ischaemia or immediately before reperfusion resulted in IA/AAR ratios (19.6 ± 6.7 and 26.5 ± 7.0%) and IA/LV ratios (9.8 ± 4.2 and

12.9 ± 4.3%) that did not differ significantly from those observed when OA-NO$_2$ was administered 15 min prior to ischaemia. There was a trend towards a more pronounced cardioprotection with the 3 days pretreatment that was not statistically significant (*Figure 4B* and *C*).

After pretreatment with PDTC, no difference in IA/AAR (18.2 ± 5.7 vs. 18.2 ± 5.8%, $P = 1.00$) and IA/LV ratio (8.4 ± 1.8 vs. 9.3 ± 3.8%, $P = 1.00$) was observed between OA-NO$_2$ and vehicle-treated animals (*Figure 4B* and *C*). No statistically significant difference was observed between OA-NO$_2$-treated animals and
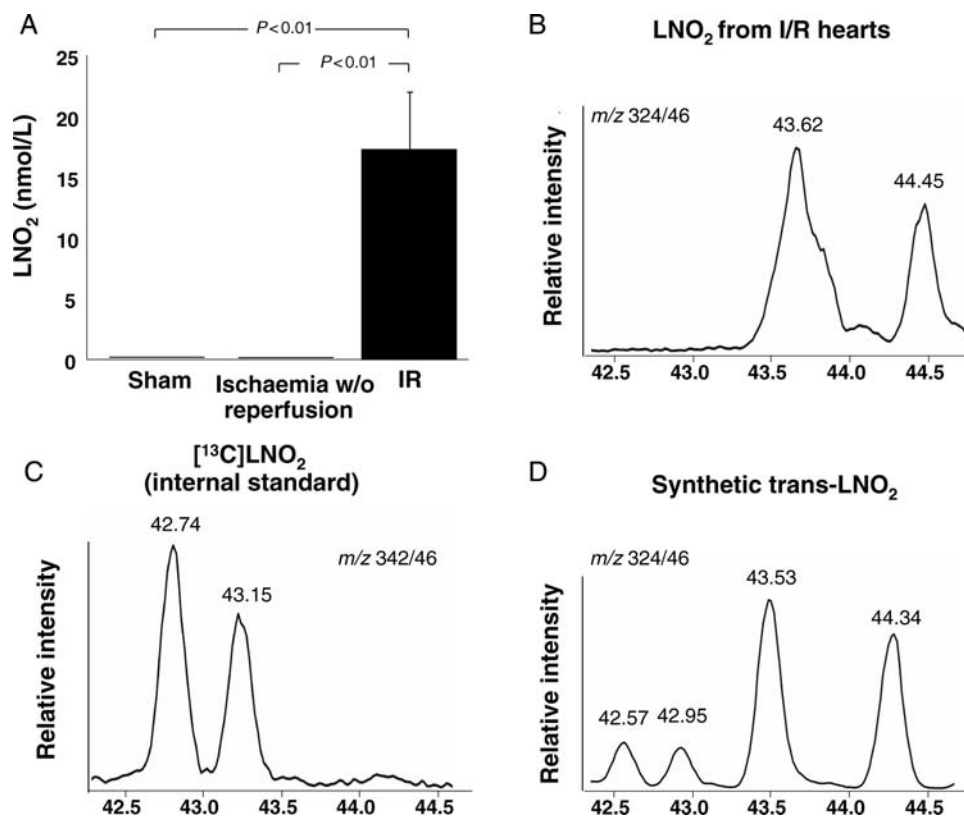
Figure 2 Endogenous generation of LNO$_2$ following ischaemia and reperfusion. (*A*) Tissue concentration of LNO$_2$ after I/R, ischaemia without reperfusion or sham surgery. (*B*) Chromatogram of LNO$_2$ extracted from mouse hearts subjected to ischaemia and reperfusion. LNO$_2$ eluted later than the [^{13}C]-labelled *cis*-LNO$_2$ which was used as internal standard (*C*) and co-eluted with synthetic *trans*-LNO$_2$ (*D*). The slight shift in retention times between chromatograms (*B* and *D*) is due to variations between separate chromatographic runs.

animals treated with PDTC and vehicle ($P = 0.21$) or animals treated with the combination of PDTC and OA-NO$_2$ ($P = 0.22$, *Figure 4B* and *C*). All treatment groups showed similar AAR/LV ratios (*Figure 4D*). Mice did not exhibit any apparent discomfort or toxic effects of PDTC or OA-NO$_2$ after 24 h of reperfusion.

3.3 Echocardiographic studies

After 24 h of reperfusion, transthoracic echocardiography revealed that mice treated with OA-NO$_2$ displayed significantly better fractional shortening than vehicle-treated mice (41.1 ± 4.0 vs. $34.7 \pm 5.1\%$, $P < 0.01$; *Figure 4E*). Mice from both groups did not exhibit increased LVEDd within 24 h of reperfusion and no differences in LVEDd were observed between groups before or after I/R (before I/R: 3.55 ± 0.07 mm vs. 3.53 ± 0.15 mm, $P = 0.98$; after I/R: 3.55 ± 0.20 mm vs. 3.51 ± 0.23 mm, $P = 0.50$). Heart rates did not differ significantly between groups during both measurements (before I/R: 420.0 ± 47.3 vs. 454.3 ± 56.7 min^{-1}, $P = 0.21$; after I/R: 427.9 ± 80.5 vs. 441.9 ± 40.1 min^{-1}, $P = 0.67$).

3.4 Activation of NFκB p65 subunit

Mice treated with OA-NO$_2$ showed markedly reduced NFκB p65 activation (*Figure 5A*). P65 activation in OA-NO$_2$-treated mice was more pronounced than in sham-operated animals. Although

differences between these two groups were not statistically significant, a trend towards residual p65 activity could be seen in OA-NO$_2$-treated animals.

3.5 *In vivo* assessment of NFκB p65 nitroalkylation

Immunoprecipitation of NFκB p65 from heart tissue of OA-NO$_2$-treated mice displayed covalently adducted OA-NO$_2$. Affinity-purified NFκB p65 fractions were treated with BME and upon LC/MS analysis revealed exchange of the OA-NO$_2$ with BME, yielding BME-OA-NO$_2$ adducts (*Figure 5B*). No BME-OA-NO$_2$ or LNO$_2$ adduct formation was detected in vehicle-treated animals, presumably because of detection sensitivity. When the NFκB p65 antibody was omitted from immunoprecipitation protocols, BME-OA-NO$_2$ adduct formation was not detectable, excluding the possibility of non-specific OA-NO$_2$ binding to agarose beads.

3.6 Downstream mediators of NFκB

OA-NO$_2$-treated animals showed significantly reduced mRNA expression for ICAM-1, MCP-1, and a reduction of mean VCAM-1 mRNA levels that was not statistically significant (*Figure 6A–C*). With the exception of MCP-1, sham-operated
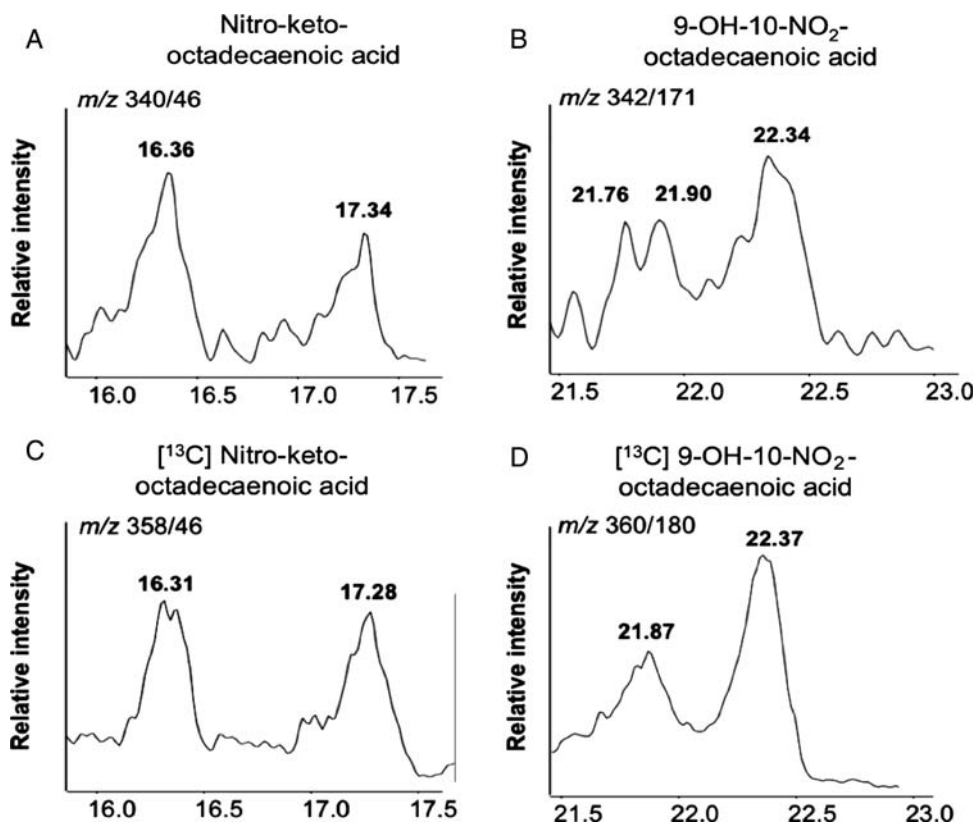
Figure 3 LNO_2 is further oxidized to keto (*A*) and hydroxyl (*B*) derivatives in murine heart tissue after I/R; and 3 μM [^{13}C]LNO_2 subjected to air oxidation overnight in Tris buffer (pH 8) was used as the precursor for oxidized ^{13}C-labelled LNO_2 derivatives. These species served as internal standards for identification of endogenously produced oxidized derivatives of LNO_2 (*C* and *D*).

mice displayed mRNA levels lower than those of OA-NO$_2$-treated mice. In the case of ICAM-1, these differences were significant.

3.7 Leukocyte infiltration and systemic inflammation

Immunofluorescence demonstrated a pronounced reduction of ICAM-1 protein expression in vessels of OA-NO$_2$-treated mice in comparison to vehicle-treated animals (*Figure 7A*). Mice treated with OA-NO$_2$ had markedly reduced neutrophil accumulation within the infarct zone compared with vehicle-treated mice as indicated by immunofluorescent staining for the neutrophil-specific GPI-anchored protein Ly-6-G (*Figure 7B*). Moreover, tissue levels of MPO, a marker of neutrophil-mediated inflammatory tissue damage, were also diminished in OA-NO$_2$-treated animals (*Figure 7C*). Serum levels of TNFα and IL-6 were also significantly lower in OA-NO$_2$-treated animals than in vehicle-treated animals (*Figure 7D* and *E*).

3.8 Assessment of apoptosis

When compared with vehicle-treated animals, OA-NO$_2$-treated mice consistently showed a significant reduction in the density and distribution of TUNEL-positive cells within the area at risk (*Figure 8A* and *B*). No TUNEL-positive apoptotic cells were observed in non-infarcted tissue. These data were confirmed by

decreased caspase 3 activity in heart tissues from OA-NO$_2$-treated animals when compared with vehicle-treated animals (*Figure 8C*).

4. Discussion

This clinically relevant *in vivo* murine model of focal ischaemic heart injury revealed the *de novo* generation of electrophilic NO$_2$-FA derivatives in reperfused ischaemic heart tissue. Motivated by *in vitro* observations of the pluripotent anti-inflammatory signalling actions of NO$_2$-FA, synthetic OA-NO$_2$ was injected intraperitoneally 15 min before or at the time of reperfusion of mice subjected to 30 min of myocardial ischaemia induced by coronary artery ligation. This resulted in (i) a significant reduction in myocardial infarct volume, (ii) preservation of left ventricular function, (iii) inhibition of NFκB activation upon post-translational modification of the p65 subunit by OA-NO$_2$, (iv) suppression of NFκB-dependent myocardial expression of the pro-inflammatory genes ICAM-1, MCP-1, and VCAM-1 and circulating TNFα and IL-6, (v) inhibition of myocardial neutrophil infiltration, and (vi) attenuation of myocyte apoptosis in the ischaemic zone.

The nitration of unsaturated fatty acids occurs endogenously and is stimulated by oxidative inflammatory conditions.[15] Cholesteryl-nitrolinoleate is formed by LPS and cytokine-activated macrophages[16] and LNO$_2$, OA-NO$_2$, and cholesteryl-nitrolinoleate
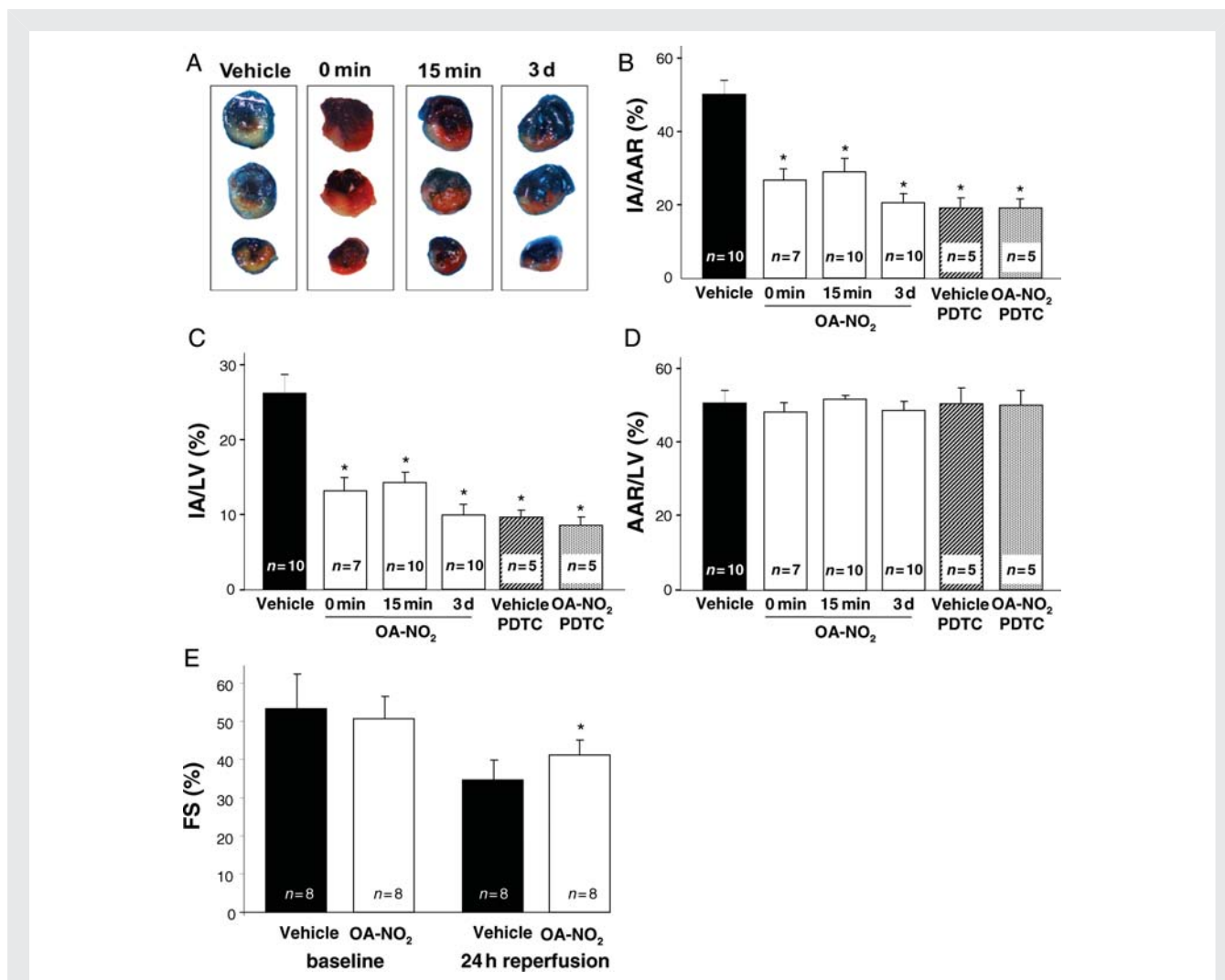
Figure 4 Effect of OA-NO$_2$ on infarct size and left ventricular function following ischaemia and reperfusion. (A) Representative images of hearts from vehicle- or OA-NO$_2$-treated animals stained with Evan's blue dye. (B) OA-NO$_2$ treatment at reperfusion, 15 min or 3 days prior to reperfusion lead to a significant reduction in infarct area/area at risk ratio when compared with vehicle-treated animals (*P < 0.01 vs. vehicle). Pretreatment with pyrrolidinedithiocarbamate (PDTC) abolished the effect of OA-NO$_2$. (C) Infarct area to left ventricular (IA/ LV) areas reflected the results observed for the IA/AAR ratio (*P < 0.01 vs. vehicle). (D) No difference in AAR/LV ratio was observed between the different treatment groups. (E) OA-NO$_2$ treatment resulted in a significantly improved left ventricular function after I/R, as assessed by transthoracic M-mode echocardiography (*P < 0.01).

have been detected in human plasma and lipoproteins.[17–20] In cardiac tissue, nitrohydroxyeicosatetraenoic acid has been measured[21] and following IPC of rat hearts on a Langendorff perfusion system, LNO$_2$ formation was observed in mitochondria.[12] Whereas these data confirm the presence of endogenous NO$_2$-FA in vivo, the present study shows the enhanced de novo generation of fatty acid nitroalkene derivatives following an in vivo pathophysiological stimulus. Since increased generation of ROS, •NO-derived species, and protein tyrosine nitration products are a hallmark of myocardial ischaemia/reperfusion injury,[2] the formation of NO$_2$-FA in this setting is consistent with nitrating and oxidizing species being generated by multiple oxidative inflammatory reactions stemming from the precursors •NO and nitrite (NO$_2^-$).[11,22]

Multiple lines of in vitro evidence support that NO$_2$-FA inhibit inflammatory signalling via •NO- and cGMP-independent mechanisms. For example, NO$_2$-FA decrease neutrophil and platelet activation and suppress nitric oxide synthase 2 expression and related inflammatory responses.[16,23] In vascular endothelium, NO$_2$-FA inhibit VCAM-1-dependent transendothelial monocyte migration[24] and potently induce HO-1 expression and activity.[25] Finally, nanomolar concentrations of NO$_2$-FA inhibit NFκB-dependent cytokine and adhesion molecule expression in cultured monocytes.[16,24]

The strongly electron-withdrawing nitro (NO$_2$) group bonded to olefinic carbons of mono- or polyunsaturated fatty acids renders the vicinal olefinic carbon strongly electrophilic. This vinyl nitro (e.g. nitroalkene) configuration supports a kinetically rapid and reversible covalent adduction of nucleophilic amino acids, primarily cysteine,
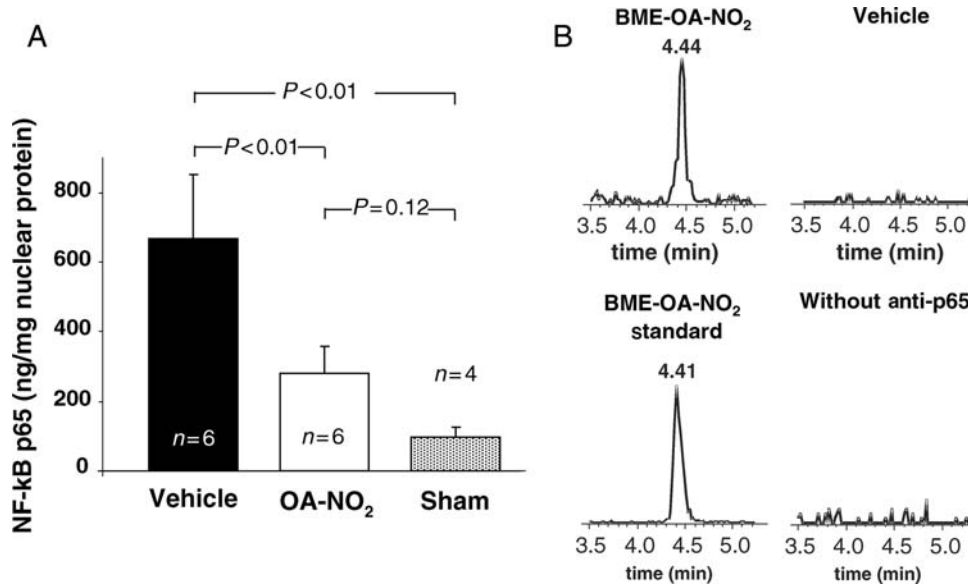
Figure 5 Inhibition of NFκB activation by OA-NO$_2$. (*A*) Reduction of NFκB p65 activity in myocardial tissue of OA-NO$_2$ treated animals following I/R. (*B*) Assessment of nitroalkylation of p65 in myocardial tissue.
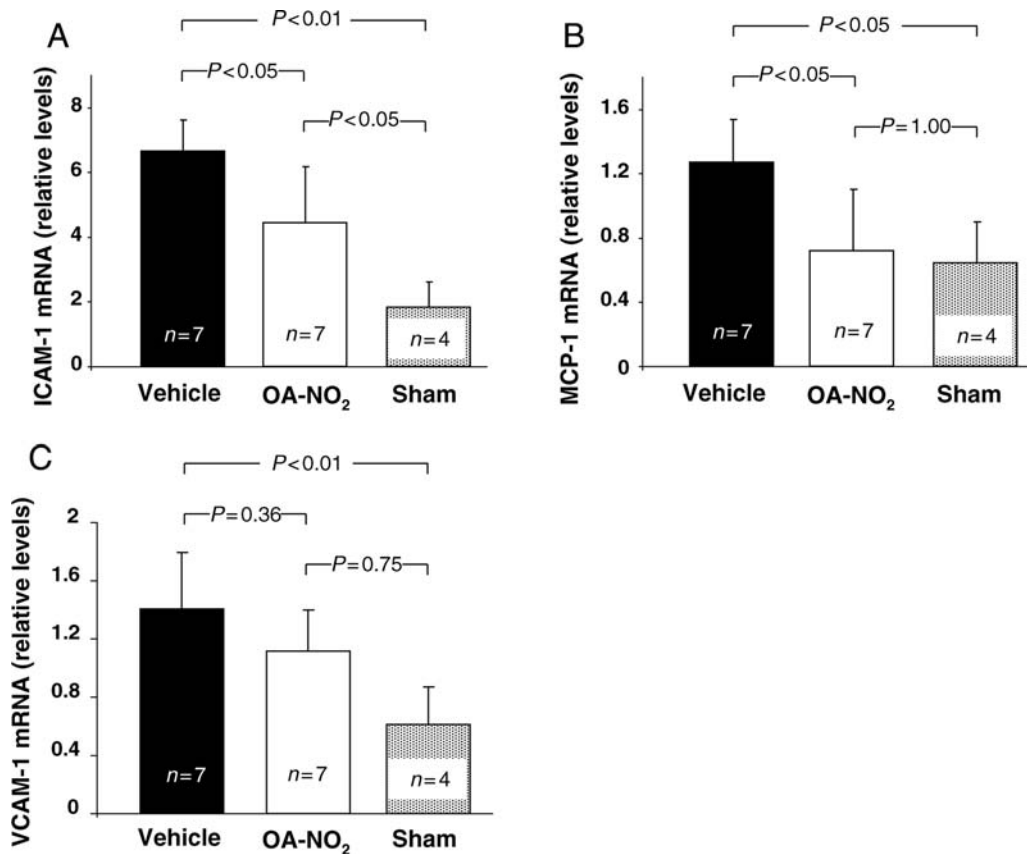

Figure 6 Effect of OA-NO$_2$ on mRNA levels of adhesion molecules and MCP-1. (*A*) Reduced NFκB activation in OA-NO$_2$-treated animals was accompanied by a significant reduction of ICAM-1 mRNA expression. (*B*) Significant suppression of MCP-1 mRNA levels in OA-NO$_2$-treated animals. (*C*) VCAM-1 mRNA levels also showed a trend towards a reduction. All values are normalized to β$_2$-microglobulin.
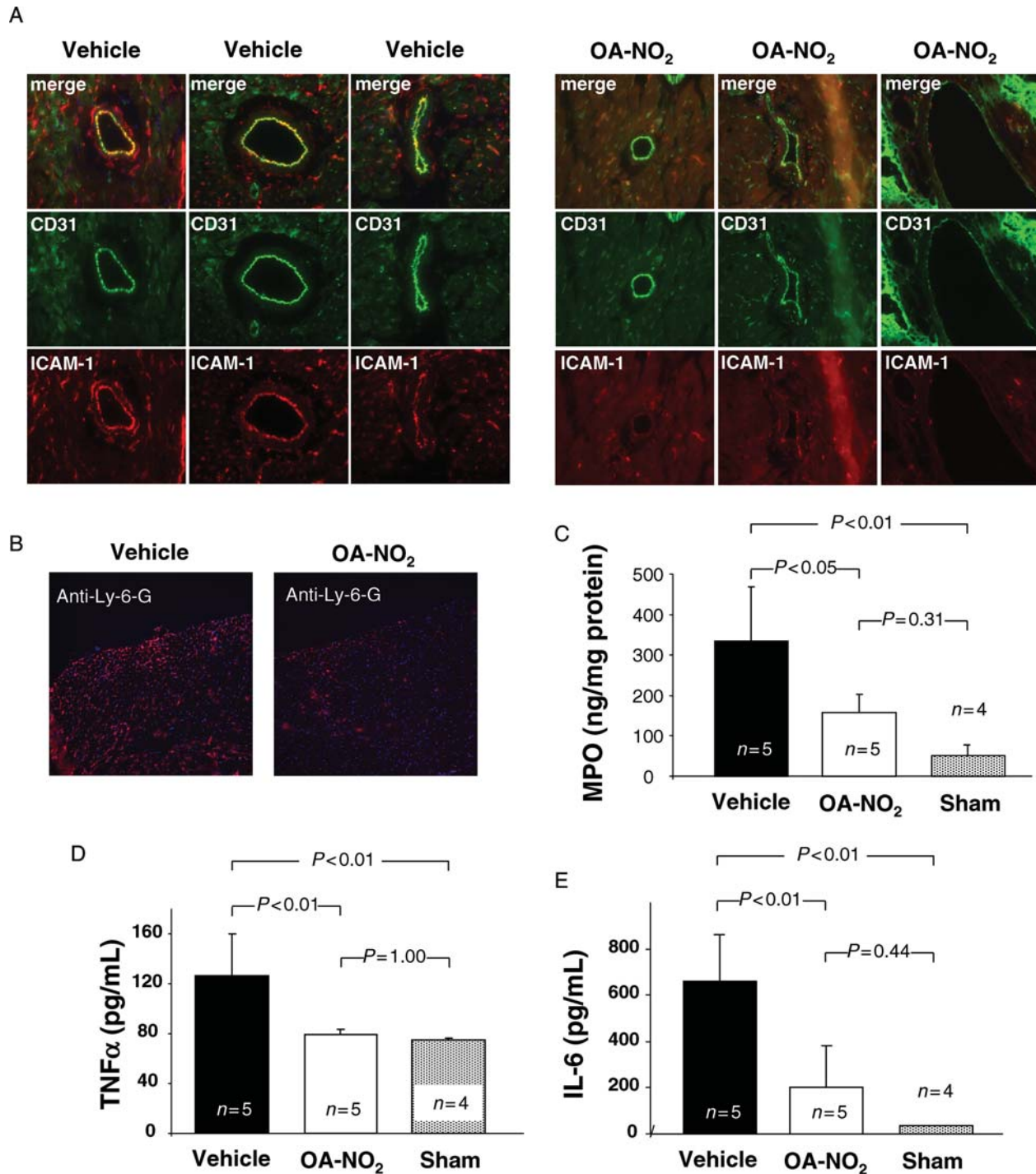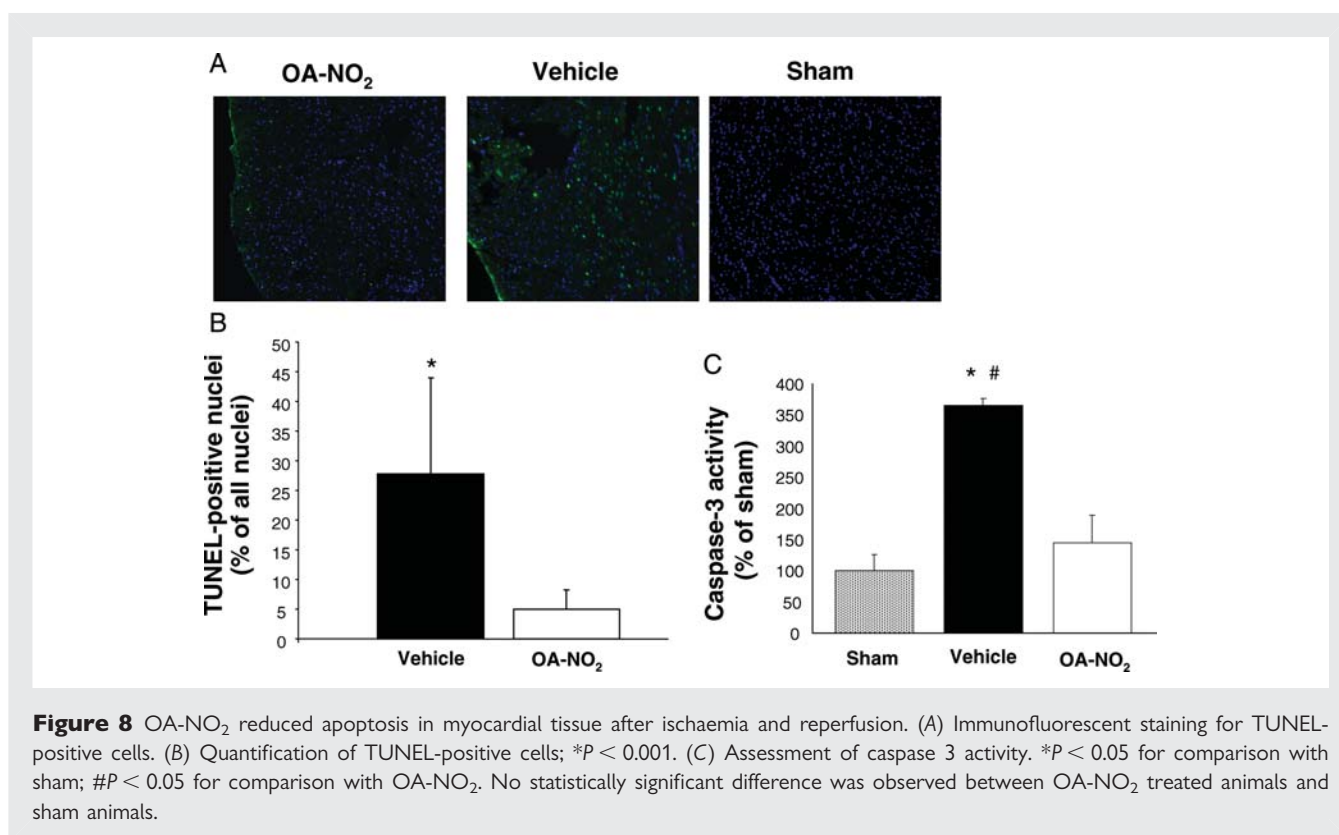
Figure 7 Effect of OA-NO$_2$ on leukocyte infiltration and systemic inflammation following ischaemia and reperfusion. (*A*) Immunofluorescent staining for ICAM-1 (red) and CD31 (green) in vessels of OA-NO$_2$ (right panel) and vehicle-treated animals (left panel). OA-NO$_2$-treated animals showed a marked reduction in ICAM-1 immunoreactivity. (*B*) Neutrophil accumulation in infarcted zone of myocardium. Immunofluorescent staining for neutrophil-specific Ly-6-G revealed increased neutrophil infiltration in vehicle-treated mice. (*C*) Determination of MPO tissue levels confirmed increased neutrophil infiltration in vehicle-treated animals. (*D*) Serum TNFα levels were significantly reduced in OA-NO$_2$ treated animals 6 h after reperfusion. (*E*) Serum IL-6 levels underwent significant reduction in OA-NO$_2$-treated mice.

Figure 8 OA-NO$_2$ reduced apoptosis in myocardial tissue after ischaemia and reperfusion. (*A*) Immunofluorescent staining for TUNEL-positive cells. (*B*) Quantification of TUNEL-positive cells; *$P < 0.001$. (*C*) Assessment of caspase 3 activity. *$P < 0.05$ for comparison with sham; #$P < 0.05$ for comparison with OA-NO$_2$. No statistically significant difference was observed between OA-NO$_2$ treated animals and sham animals.

and to a lesser extent lysine and histidine residues.[24,26,27] Current data support that many inflammatory-related transcription factors possess electrophile-reactive thiols that are functionally significant and thus contribute to the transduction of stress-related signalling reactions. This property facilitates the post-translational modification of various signalling mediators and transcription factors by NO$_2$-FA, including the activation of PPARγ[28,29] and Nrf2/Keap1[30] as well as the inhibition of NFκB by nitroalkylation of the NFκB p65 subunit.[24]

The myocardial protection lent by OA-NO$_2$ administration in this model of I/R was mediated in part by inhibition of NFκB, although it is noted that there may have been some residual nuclear translocation of p65 in OA-NO$_2$-treated animals when compared with sham animals. The precept that OA-NO$_2$-induced NFκB inhibition is reinforced by the observation that inhibition of NFκB signalling by PDTC paralleled OA-NO$_2$-mediated reduction in infarct size. PDTC, while not absolutely specific as a p65-directed reagent, has been used in similar studies as an inhibitor of NFκB.[31-33] The slightly greater extent of protection lent by PDTC may be a consequence of a higher dose of PDTC being used and its administration at an earlier time point, 30 min prior to the surgery. Inhibition of NFκB signalling by NO$_2$-FA is mediated by post-translational modification of the p65 subunit, an event contributing to reductions in ICAM-1, MCP-1, and VCAM-1 expression, neutrophil activation, and apoptosis. It remains possible that OA-NO$_2$ also affects pro-inflammatory gene expression and cellular responses via mechanisms independent of NFκB regulation.

Administration of OA-NO$_2$ reduced the extent of apoptosis in ischaemic myocardium, an effect that can also be a consequence of inhibition of NFκB activation. Whereas myocyte apoptosis plays a

central role in the development of myocardial reperfusion injury,[1] cellular necrosis may also significantly contribute to pathogenic events. It is also possible that apoptosis and necrosis are subject to common regulatory events such as changes in the mitochondrial permeability transition pore (mPTP).[34] In this context, it is notable that NO$_2$-FA covalently adduct the adenine nucleotide translocase, one of the components associated with the mPTP.[12] Future studies can address whether NO$_2$-FA inhibit both apoptosis and necrotic cell death via interference of mPTP assembly or function.

Current insight indicates that a complex array of events will occur when electrophilic species react with signalling mediators, thus the biological effects of OA-NO$_2$ observed herein are not restricted to just one proposed linear mechanism of action. It is possible that, in addition to inhibition of NFκB activation,[35-37] other events such as activation of Nrf2/Keap1 and subsequent upregulation of HO-1 expression, PPAR-γ activation, mitochondrial uncoupling, heat shock factor activation, and other actions of NO$_2$-FA also can contribute to myocardial protection.

The formation of OA-NO$_2$ and LNO$_2$ during reperfusion, combined with the profound protective effects shown upon exogenous administration of OA-NO$_2$ indicates that fatty acid nitration yields an endogenous mediator capable of limiting the damage induced by tissue reoxygenation. Its generation is a consequence of the same process central to reoxygenation-induced tissue damage, the formation and reactions of oxidative inflammatory mediators. In this context, it is important to note that NO$_2$-FA generation was not observed when myocardial ischaemia occurred without reperfusion. Notably, no differences in extents of fatty acid nitration were observed with varying periods of ischaemia. Longer periods

of ischaemia, and thus longer periods of reducing conditions in the tissue, might conceivably lead to increased formation of mediators of fatty acid nitration upon reintroduction of oxygen. A possible explanation for no differences in tissue NO_2-FA content with varying ischaemia could be that the formation of precursors for fatty acid nitration reaches a steady-state concentration by 15 min and that thus upon reperfusion similar amounts of reactants are available for fatty acid nitration.

The profile of NO_2-FA generation observed *in vivo* differed from that induced by IPC in the isolated rat heart.[12] Thus, while OA-NO_2 formation was observed in the blood-reperfused heart, no changes were detected following IPC. Furthermore, IPC resulted in the generation of *cis*-LNO$_2$, whereas the present study revealed a predominant generation of *trans*-LNO$_2$ *in vivo*. Several reasons could account for these differences. For example, activation of phospholipase A$_2$ during IPC leads to preferential release of LNO$_2$ rather than OA-NO_2 from the membrane.[38] In contrast, apoptotic and necrotic cell death observed during the prolonged ischaemia in the course of I/R might lead to a different profile of fatty acid liberation.[39] Moreover, the absence of red cells, blood-borne inflammatory cells, plasma proteins, and other possible mediators results in a chemically different milieu in the isolated heart both during the ischaemic episode and during reperfusion. In particular, the predominant generation of *trans*-LNO$_2$ by blood-perfused murine heart tissue, when compared with the *cis*-LNO$_2$ noted in ischaemic preconditioned Langendorff hearts, might be explained by decreased levels of protein- and non-protein-thiols in Krebs−Henseleit buffer-perfused hearts. Thiol adduction confers a loss of the *cis−trans* configuration of NO_2-FA, with preliminary observations indicating that upon the reverse reaction, de-alkylation favours NO_2-FA release in the *trans* configuration. These reactions require further study to define potential roles determining *cis−trans* configuration.

Although the present data support a therapeutic response to acutely administered OA-NO_2 within the first 24 h of reperfusion, the long-term effects upon treatment with OA-NO_2 cannot be delineated from this study. With regard to clinical relevance, however, the timing and beneficial tissue response to OA-NO_2 when administered even at the time of reperfusion is of relevance to a clinical setting, such as the presentation of a patient with an acute myocardial infarction before angioplasty.

In conclusion, this study demonstrates *de novo* fatty acid nitration *in vivo* following a pathophysiological stimulus. We further show that OA-NO_2 supplementation exerts potent anti-inflammatory and tissue-preserving actions in the early phase of myocardial reperfusion injury. Overall, these data reveal that inflammatory-derived fatty acid nitration products mediate tissue-adaptive signalling reactions *in vivo*.

Supplementary material

Supplementary Material is available at *Cardiovascular Research* online.

Conflict of interest: B.A.F. acknowledges financial interest in Complexa, Inc.

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