

FENOIBRATE AND  
ENDOTHELIAL  
PROTECTIONFenofibrate protects endothelial  
cells against the harmful effects  
of TNF-alpha

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## INTRODUCTION

Fenofibrate, a fibric acid-derivative and peroxisome proliferator-activated receptor-alpha (PPAR $\alpha$ ) agonist, is best known for its high density lipoprotein cholesterol increasing and triglyceride reducing effects.<sup>(1,2)</sup> In addition to its anti-dyslipidaemic actions, fenofibrate has been shown to exert several pleiotropic (non-lipid) vasculo-protective effects, including antioxidant,<sup>(3-6)</sup> anti-thrombotic,<sup>(7,8)</sup> and anti-apoptotic effects.<sup>(9,10)</sup>

Many of the beneficial pleiotropic effects of fenofibrate on the vasculature are thought to be driven by increased endothelium-dependent nitric oxide (NO) release, as demonstrated in a study which showed that fenofibrate treatment improved hyperaemia-induced flow-mediated dilatation in patients with hypertriglyceridaemia.<sup>(1)</sup> NO is an important endothelium-derived molecule involved in the maintenance of vascular homeostasis,<sup>(11)</sup> and is synthesised by the NO synthase (NOS) enzyme family, viz. endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS).<sup>(12)</sup> In addition to its NO modulatory effects, fenofibrate has been shown to possess antioxidant properties. For example, fenofibrate reduced superoxide levels and apoptosis in human umbilical vein endothelial cells (HUVECs) exposed to high glucose treatment,<sup>(10)</sup> and increased superoxide dismutase activity in rat brain microvessels.<sup>(3)</sup>

## ABSTRACT

**Introduction:** Fenofibrate exerts pleiotropic effects on endothelial cells (ECs) by, amongst others, increasing nitric oxide (NO) production. We aimed to investigate fenofibrate's putative beneficial actions in healthy or TNF-alpha-induced dysfunctional ECs.

**Methods:** Fenofibrate-induced pro-vasodilatory responses were assessed in aortic rings (50 - 125 $\mu$ M; 30min) with and without L-NMMA (100 $\mu$ M). Rat cardiac microvascular ECs were treated with fenofibrate (30 and 50 $\mu$ M; 1h). In the pre-treatment experiments, fenofibrate (50 $\mu$ M) was administered one hour before TNF-alpha treatment (20ng/ml; 24h). NO-production (DAF-2/DA or Griess assay), mitochondrial ROS-production (MitoSox<sup>TM</sup>), cell viability (propidium iodide staining), and changes in the expression/phosphorylation of critical endothelial proteins were measured by Western blotting.

**Results:** Fenofibrate increased NO-production ~2-fold in healthy ECs (p<0.05 vs. vehicle). A ~23% pro-vasodilatory response was induced in aortic rings, which was reversed by L-NMMA (p<0.05 vs. fenofibrate). Fenofibrate pre-treatment ameliorated TNF-alpha-induced endothelial dysfunction by reversing the loss of NO, improving oxidative stress, restoring cell viability and preventing caspase-3 activation. Protective effects were underpinned by ~47% and ~49% up-regulation of activated eNOS and AMP-kinase, respectively (p<0.05 vs. TNF-alpha).

**Conclusions:** Fenofibrate protects TNF-alpha-induced dysfunctional ECs via up-regulated eNOS-NO, reduced oxidative stress and improved cell viability. These novel findings warrant further investigations to explore the potential use of fenofibrate as an anti-endothelial dysfunction therapeutic agent. SAHeart 2017;14:22-34

Cardiovascular risk factors, such as diabetes mellitus and obesity, as well as atherosclerosis are associated with inflammation and increased levels of pro-inflammatory cytokines.<sup>(13-15)</sup> Endothelial cells are important cellular targets of pro-inflammatory cytokines such as tumour necrosis factor alpha (TNF-alpha),<sup>(16)</sup> and exposure of the endothelium to TNF-alpha can result in endothelial dysfunction.<sup>(13)</sup> In fact, TNF-alpha is considered one of the main promoters of endothelial dysfunction during the early stages of atherosclerosis.<sup>(17)</sup> Hence, investigations into potential therapeutic interventions that can prevent TNF-alpha

induced vascular damage are important. Endothelial dysfunction is characterised by reduced NO bioavailability and impaired endothelium-dependent vasodilation<sup>(18)</sup> and is regarded as an early, reversible precursor and predictor of atherosclerosis.<sup>(19-20)</sup> Although the exact cellular mechanisms underlying TNF- $\alpha$  induced endothelial dysfunction are still under scrutiny,<sup>(22)</sup> oxidative stress is generally recognised as the candidate common mechanism.<sup>(23-24)</sup>

In view of the fact that endothelial dysfunction is potentially reversible, early therapeutic interventions could prevent future cardiovascular disease. Previous studies have investigated the effect of fenofibrate on TNF- $\alpha$  induced changes in the vasculature. Zhao and Wu (2004) showed that fenofibrate reduced TNF- $\alpha$  levels in the serum of rabbits on a high cholesterol diet as well as TNF- $\alpha$  secretion from adipocytes.<sup>(26)</sup> Furthermore, fenofibrate pre-treatment prior to TNF- $\alpha$  administration in HUVECs was able to inhibit TNF- $\alpha$  induced expression of cluster of differentiation 40 (CD40).<sup>(27-28)</sup> Huang, et al. (2009) showed that fenofibrate prevented increases in monocyte adhesion to endothelial cells in TNF- $\alpha$ -stimulated human aortic endothelial cells.<sup>(29)</sup> However, as far as we are aware, no previous studies have investigated whether fenofibrate can protect vascular endothelial cells against the harmful effects of TNF- $\alpha$  with regards to NO production, ROS generation and cell viability. Therefore, the present study aimed to investigate whether pre-treatment with fenofibrate protects adult rat endothelial cells against TNF- $\alpha$  induced endothelial dysfunction, and if so, whether the protection is associated with the modulation of NO biosynthesis, ROS generation and markers of cell death.

## MATERIALS AND METHODS

### Research materials and reagents

Endothelial growth medium (Endothelial cell basal medium-2; EBM-2) was purchased from Clonetics (Cambrex Bio Science, Walkersville, USA); 4,5-Diaminofluorescein diacetate (DAF-2/DA) and N<sup>G</sup>-Monomethyl-L-arginine monoacetate (L-NMMA) were from Calbiochem (San Diego, California, USA). Attachment factor, trypsin (500 BAEE units trypsin / 180 $\mu$ g EDTA•4Na per ml in Dulbecco's PBS), dihydroethidium (DHE) and MitoSox<sup>TM</sup> Red were from Life Technologies (Carlsbad, California, USA). Fenofibrate, human recombinant TNF- $\alpha$ , dihydrorhodamine-123 (DHR-123), dimethoxy-1, 4-naphthoquinone (DMNQ), diethylamine NONOate (DEA/NO), dimethyl sulfoxide (DMSO), acetylcholine, phenylephrine and Griess Reagent were from Sigma-Aldrich (St Louis, Missouri, USA). The following antibodies were from Cell Signaling Technologies (Beverly, Massachusetts, USA): eNOS, phosphorylated-eNOS (Ser 1177); Akt, phosphorylated Akt (Ser 473); AMP activated protein kinase (AMPK), phosphorylated AMPK (Thr 172); cleaved caspase-3 and I $\kappa$ B $\alpha$ . p22PHOX, and

nitrotyrosine antibodies were purchased from Santa Cruz Biotechnologies. Propidium iodide (PI) solution and cell staining buffer were purchased from Bio-Legend (Biotec-Biotech, San Diego, California, USA). Enhanced chemiluminescence (ECL) detection reagent, ECL hyperfilm, and Horseradish peroxidase-linked anti-rabbit IgG were from AEC Amersham (Buckinghamshire, UK). Polyvinylidene difluoride (PVDF) membrane (Immobilon<sup>TM</sup>-P) and authentic peroxyxynitrite were from Millipore (Bedford, MA, USA). Foetal bovine serum was purchased from Highveld Biological (Lyndhurst, South Africa). All other chemicals and buffer reagents were purchased from Merck (Damstadt, Germany).

### Adult rat aortic ring studies

#### Animal care

Experiments were conducted according to the "Guide for the care and use of laboratory animals" published by the US National Institutes of Health (NIH publication no. 85 - 23, revised 1985) and the South African Bureau of Standards (SANS 10386, 2008). Ethics approval was received from the University of Stellenbosch (Project number: SU-ACUM11 - 00002). Male Wistar rats weighing 200 - 250g were supplied by the Central Research Facility of the Faculty of Medicine and Health Sciences of Stellenbosch University where they were housed in temperature controlled conditions (22 - 24°C). Animals were subjected to a 12 hour light and dark cycle and had free access to standard rat chow and water. Animals were anaesthetised with pentobarbital (160mg/kg) until deep anaesthesia was reached as evidenced by the lack of a pedal reflex. They were then euthanised by exsanguination before excision of the aortas.

#### Assessment of aortic vasoactive responses

The chest cavity was opened by incision and thoracic aorta excised and placed in ice cold Krebs-Henseleit solution, containing sodium chloride (NaCl) 119mM, sodium bicarbonate (NaHCO<sub>3</sub>) 25mM, potassium chloride (KCl) 4.75mM, monopotassium phosphate (KH<sub>2</sub>PO<sub>4</sub>) 1.2mM, magnesium sulfate heptahydrate (MgSO<sub>4</sub>•7H<sub>2</sub>O) 0.6mM, sodium sulfate (Na<sub>2</sub>SO<sub>4</sub>) 0.6mM, calcium chloride monohydrate (CaCl<sub>2</sub>•H<sub>2</sub>O) 1.25 mM, and glucose 10mM. Excess perivascular fat and connective tissue were removed. Aortas were cut into 3 - 4mm ring segments that were subsequently mounted onto 2 steel hooks in a 25ml organ bath (AD Instruments, Bella Vista, New South Wales, Australia) containing oxygenated (95% O<sub>2</sub> and 5% CO<sub>2</sub>) Krebs-Henseleit buffer at 37°C. Aortic ring tension was recorded with an isometric force transducer (TRI202PAD, Panlab, IComellà, Barcelona, Spain) and data analysed with LabChart 7 software (Dunedin, New Zealand). The isometric tension measurement protocol was based on a modification of a previously described technique.<sup>(29)</sup> Briefly, mounted aortic rings were stabilised under a resting tension of 1.5g during

which time the Krebs-Henseleit buffer in the organ bath was changed every 10 minutes for 30 minutes with pre-warmed Krebs-Henseleit solution. Following this, all aortic rings were pre-contracted with phenylephrine administration (1 $\mu$ M) to the organ bath until the maximum contraction tension was reached. Subsequently, aortic rings were randomly divided into groups that either received cumulative concentrations of the known pro-vasodilatory agent, acetylcholine (30nM - 10 $\mu$ M), or cumulative concentrations of fenofibrate (50 $\mu$ M - 125 $\mu$ M). Pharmacological NOS inhibition was achieved by adding L-NMMA (100 $\mu$ M) to the water bath 15 minutes before the phenylephrine-fenofibrate protocol.

## Endothelial cell studies

### Cell cultures

Primary adult rat cardiac microvascular endothelial cells (CMECs) and aortic endothelial cells (AECs) were purchased from VEC Technologies (Rensselaer, New York, USA). CMECs and AECs were maintained in EBM-2, supplemented with 10% foetal bovine serum, growth factors and antibiotics as per the supplier's instructions. CMEC and AEC subcultures were passaged to a maximum of 4 generations in 35mm attachment factor-coated petri dishes. All cultures received fresh growth medium every second day. Endothelial cell culture purity was regularly monitored by light microscopic validation of the typical monolayer, cobblestone appearance exhibited by cultured endothelial cells<sup>(30,31)</sup> as well as confirmation by flow cytometric analysis of >80% positive staining with the endothelial cell-specific marker, acetylated low density lipoprotein labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (Dil-ac-LDL; Biomedical Technologies, Stoughton, Massachusetts, USA).<sup>(30,31)</sup> All cell cultures were grown until 100% confluency was reached for experimental purposes.

### Experimental conditions and procedures

Cultured CMECs (fully confluent) were washed once with phosphate buffered saline (PBS) prior to treatment with fenofibrate or vehicle solution. Fenofibrate was dissolved in 0.2% DMSO. Cells were treated with 30 or 50 $\mu$ M fenofibrate or 0.2% DMSO for 1 hour. Untreated, time-matched control samples received fresh medium only. In a separate set of investigations, the putative protective effects of fenofibrate pretreatment were explored in TNF-alpha-treated CMECs. Briefly, CMECs were washed once with PBS, pretreated with fenofibrate (50 $\mu$ M) for 1 hour, after which TNF-alpha (20ng/ml) was administered to the fenofibrate-pretreated cells for an additional 24 hours without changing the growth medium. Separate CMEC samples were treated with fenofibrate for 25 hours or TNF-alpha for 24 hours respectively. Control samples were incubated with growth medium, and vehicle control samples with 0.2% DMSO (vehicle) in a time-matched fashion. After treatment, cells were washed twice with PBS and prepared for flow cytometric, Griess or Western blot analyses.

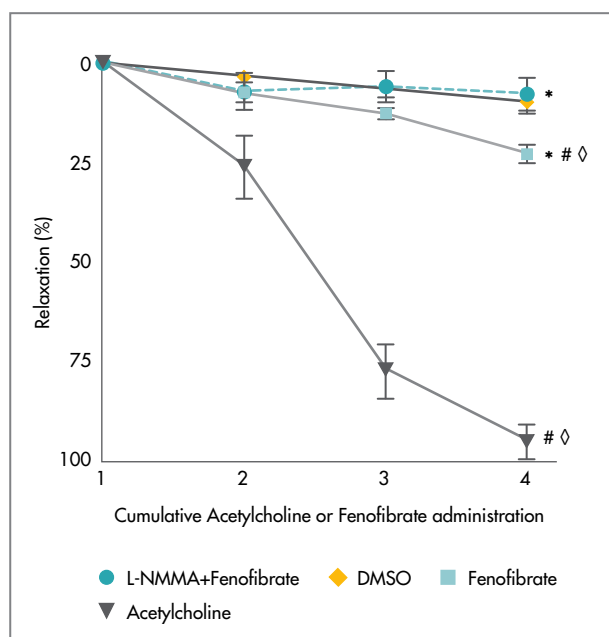
### Flow cytometric analyses

Intracellular levels of NO, superoxide and peroxynitrite were determined by flow cytometric analysis of the mean fluorescence intensity generated with the fluorescent probes: DAF-2/DA (10  $\mu$ M; sensitive for NO), DHE (5 $\mu$ M; sensitive for superoxide), DHR-123 (2 $\mu$ M; sensitive for peroxynitrite), and MitoSox™ (5 $\mu$ M; sensitive for mitochondrial ROS). The flow cytometry-based assays used in this study were previously extensively characterised and validated.<sup>(32,33)</sup> Briefly, after the respective treatment periods as described above, cultured cells were washed twice with PBS and incubated at 37°C for an additional 2 hours with DAF-2/DA, DHE or DHR-123, or 15 minutes with MitoSox™ to allow the fluorescent probes to react with their respective intracellular target molecules. Following this, the fluorescent probe-containing cells were washed once with PBS and removed from culture by trypsinization. Trypsinized cells were centrifuged for 3 minutes at 1 000rpm and the pellet subsequently re-suspended in PBS in preparation for flow cytometric analysis. To verify the sensitivity of the fluorescent probes for their target molecules, positive control experimental groups were included in which cells were treated with 100 $\mu$ M of the NO-donor, DEA/NO (for DAF-2/DA sensitivity), 10 $\mu$ M of the superoxide donor, DMNQ (DHE sensitivity), or 100 $\mu$ M authentic peroxynitrite (for DHR-123 sensitivity) respectively, as previously described.<sup>(32-34)</sup> All flow cytometric analyses were performed with a Becton Dickinson FACSCalibur® (Becton, Dickinson and Co, San Jose, California, USA) using WinMDI® version 2.9 software (Purdue University Cytometry Laboratories, West Lafayette, USA). Acquisition was set at 10 000 events/sample, and the mean fluorescence intensity of DAF-2/DA and DHE was analysed in flow channel 1 and flow channel 2 respectively. DHR-123 and MitoSox™ were analysed in flow channel 3.

Loss of cell viability was measured by means of flow cytometric analysis of propidium iodide staining according to the manufacturer's instructions. Trypsinized cells were incubated with propidium iodide for 15 minutes in the dark before flow cytometric analysis. Samples were analysed in flow channel 2 and 5 000 events were acquired per sample. Data analysis was performed with WinMDI® version 2.9 software.

### Plate reader analyses with the Griess Reagent

In order to validate the results obtained with the DAF-2/DA assay, a different NO detection method, the Griess assay, was employed to measure the cellular release of nitrites (breakdown products of NO). CMECs and AECs were cultured in 24-well microplates until confluent. Cells were treated with 50 $\mu$ M fenofibrate in 500 $\mu$ l PBS for 1 hour, after which an equal volume of 500 $\mu$ l Griess reagent<sup>(35)</sup> was added to each well. After 15 minutes incubation, the colorimetric reaction was measured at 540nm on a FLUOstar Omega platereader from BMG Labtech (Ortenberg, Germany). Serial dilutions of a nitrite



**FIGURE 1: Aortic ring relaxation induced by the administration of cumulative concentrations of fenofibrate.**

Fenofibrate administered cumulatively (1:0 $\mu$ M; 2:50 $\mu$ M; 3:100 $\mu$ M and 4:125 $\mu$ M) induced a vasodilatory response in phenylephrine pre-contracted aortic rings, which was attenuated by pre-incubation with the non-selective NOS inhibitor L-NMMA (100 $\mu$ M). Acetylcholine administration (cumulative concentrations: 1:0 $\mu$ M; 2:0.03 $\mu$ M; 3:1 $\mu$ M and 4:10 $\mu$ M) served as a positive control of maximum endothelium-dependent pro-vasodilatory response, and DMSO (cumulative concentrations: 1:0%; 2:0.02%; 3:0.04% and 4:0.05%) was the vehicle control. n=4/group.

\* =  $p < 0.05$  vs. acetylcholine, # =  $p < 0.05$  vs. L-NMMA + Fenofibrate, ◊ =  $p < 0.05$  vs. DMSO.

standard (NaNO<sub>2</sub> dissolved in PBS; 0 - 10 $\mu$ M) were included in each microplate to create a standard curve. Data were calculated from the standard curve and expressed as  $\mu$ M nitrites.

### Western Blot analyses

CMECs were lysed using a lysis buffer (20.0mM Tris-HCl; 1.0mM EGTA; 1.0mM EDTA; 150mM NaCl; 1.0mM  $\beta$ -glycerophosphate; 2.5mM sodium pyrophosphate; 0.1% sodiumdodecylsulphate; 1.0mM sodium orthovanadate; 1.0% Triton-X100; 10 $\mu$ g/ml leupeptin; 10 $\mu$ g/ml aprotinin; 50nM sodium fluoride; 50 $\mu$ g/ml phenylmethylsulfonyl fluoride) and homogenised with 0.5mm zirconium beads and a Bullet Blender™ (Next Advance, Inc., New York, USA). Protein content was determined with the Bradford assay.<sup>(36)</sup> Equal amounts of protein (20 $\mu$ g) were separated on SDS-polyacrylamide gels and electro-transferred to a polyvinylidene difluoride membrane. Non-specific protein binding sites on the membrane were blocked with 5% fat free milk powder, dissolved in tris buffered saline-Tween-20 (0.1%) for 1 hour.

Membranes were incubated overnight with the following primary antibodies: eNOS and phosphorylated-eNOS (Ser 1177), Akt and phosphorylated-Akt (Ser 473), AMPK and phosphorylated-AMPK (Thr 172),  $\kappa$ B $\alpha$ , cleaved caspase-3, nitrotyrosine and p22PHOX. The secondary antibody was horseradish peroxidase-linked anti-rabbit IgG or anti-mouse IgG and protein bands were visualised with the ECL™ chemiluminescent system. Films were densitometrically analysed (UN-SCAN-IT, Silk Scientific, Orem, UT, USA). Ponceau stains were used as loading control. The ponceau stain was densitometrically analysed and quantified. All data were then expressed as a ratio of total protein over the quantified ponceau value for that specific lane in the blot. The relative phosphorylation status of proteins was expressed as a ratio of phosphorylated over total protein expression. Membranes were stripped of primary and secondary antibodies with 0.2M NaOH and re-probed with a different antibody; therefore some proteins shared ponceau loading controls.

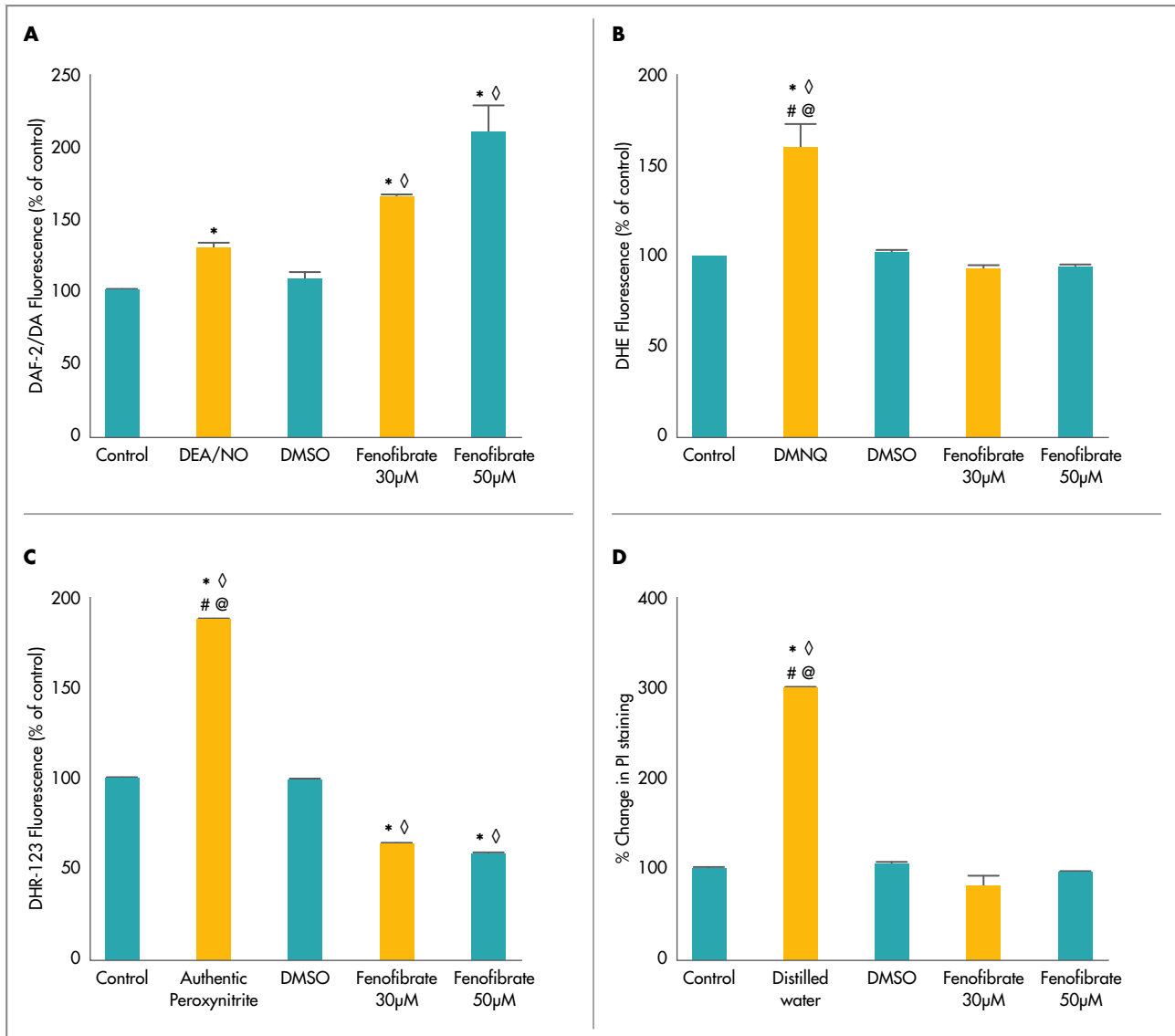
### Statistical analysis

All flow cytometry data were calculated as mean  $\pm$  standard error of the mean, with values expressed as % of control (control adjusted to 100%). Propidium iodide data were expressed as % change in non-viable (stained) cells compared to control (control adjusted to 100%). Total sample sizes for the flow cytometry investigations were derived from experiments repeated at least 2 - 3 times on separate days using cells cultured from different batches (total n-values are shown below each graph). For western blot data, controls were adjusted to 1 and sample sizes were a minimum of n=3/group. Student's t-tests or one-way analysis of variance (with Bonferroni multiple comparison test or Dunnet post hoc test) were used to determine statistical significance. All aortic ring isometric tension data were expressed as the % relaxation of maximum contraction and statistically analysed by means of two-way analysis of variance followed by Bonferonni post-test. Differences with a p value  $< 0.05$  were considered statistically significant. Data were analysed using GraphPad Prism 5 software (GraphPad Software, San Diego, California, USA). Sample sizes are indicated below each graph.

## RESULTS

### Cumulative NOS-dependent relaxation of aortic rings

Cumulative administration of fenofibrate induced a maximum vasodilatory response of 23% in phenylephrine pre-contracted aortic rings after 30 minutes (vs. DMSO vehicle: 10% relaxation;  $p < 0.05$ ) (Figure 1). Pretreatment with the NOS inhibitor, L-NMMA (100 $\mu$ M), significantly attenuated the fenofibrate-induced relaxation from 23% - 8% ( $p < 0.05$  vs. fenofibrate), thereby confirming NOS-dependence.



**FIGURE 2: Fenofibrate treatment (1 hour) in healthy CMECs.**

A. Intracellular NO production increased in both 30µM and 50µM fenofibrate treatment groups as measured by DAF-2/DA fluorescence. The NO-donor, DEA / NO (100µM) was used as positive control. Data expressed as % of control (control adjusted to 100%); n=4 - 9/group.  
 B. Intracellular superoxide production remained unchanged compared to control as measured by DHE fluorescence. The superoxide donor, DMNQ (10µM) was used as positive control. Data expressed as % of control (control adjusted to 100%); n=6 - 14/ group.  
 C. Intracellular peroxynitrite production decreased in both 30µM and 50µM fenofibrate treatment groups as measured by DHR-123 fluorescence. Authentic peroxynitrite (100µM) was used as positive control. Data expressed as % of control (control adjusted to 100%); n=7 - 8/group.  
 D. Cell viability as measured by propidium iodide staining was not affected by fenofibrate. Brief exposure (5 minutes) of the cells to osmotic stress by incubation with distilled water served as positive control. Data expressed as % change in propidium iodide staining cells from control (control adjusted to 100%); n=8 - 15/group.

\* =  $p < 0.05$  vs. control, ◇ =  $p < 0.05$  vs. DMSO, # =  $p < 0.05$  vs. fenofibrate 30µM, @ =  $p < 0.05$  vs. fenofibrate 50µM.

**Fenofibrate treatment increases intracellular NO and nitrite concentration, and decreases ROS levels, with no effect on cell viability**

The NO-donor, DEA/NO significantly increased DAF-2/DA fluorescence, confirming the NO-sensitivity of the probe (Figure 2a). At 1 hour, both 30µM and 50µM fenofibrate increased DAF-2/DA fluorescence by 1.7-fold and 2.2-fold

respectively vs. control ( $p < 0.05$ ) (Figure 2a). The superoxide-donor, DMNQ, significantly increased DHE fluorescence compared to control; however, fenofibrate exerted no effects on DHE fluorescence at either 30µM or 50µM treatment (Figure 2b). Authentic peroxynitrite significantly increased DHR-123 fluorescence compared to control. Fenofibrate treatment resulted in decreased DHR-123-fluorescence com-

pared to control at both 30 $\mu$ M (36% decrease;  $p < 0.05$ ) and 50 $\mu$ M (42% decrease;  $p < 0.05$ ) (Figure 2c). Cell viability in CMECs were not affected by 1 hour fenofibrate (30 and 50 $\mu$ M) treatment (Figure 2d). DEA/NO increased the concentration of nitrites by 2-fold compared to control ( $p < 0.0001$ ) in CMECs (Figure 3a); similarly, 1 hour treatment with 50 $\mu$ M fenofibrate

increased the concentration of nitrites by 2-fold compared to control ( $p < 0.001$ ) (Figure 3b), and by 60% in AECs ( $p < 0.05$ ) (Figure 3c).

### Fenofibrate pre-treatment prevents TNF-alpha induced effects on NO and ROS synthesis, and prevents loss of cell viability

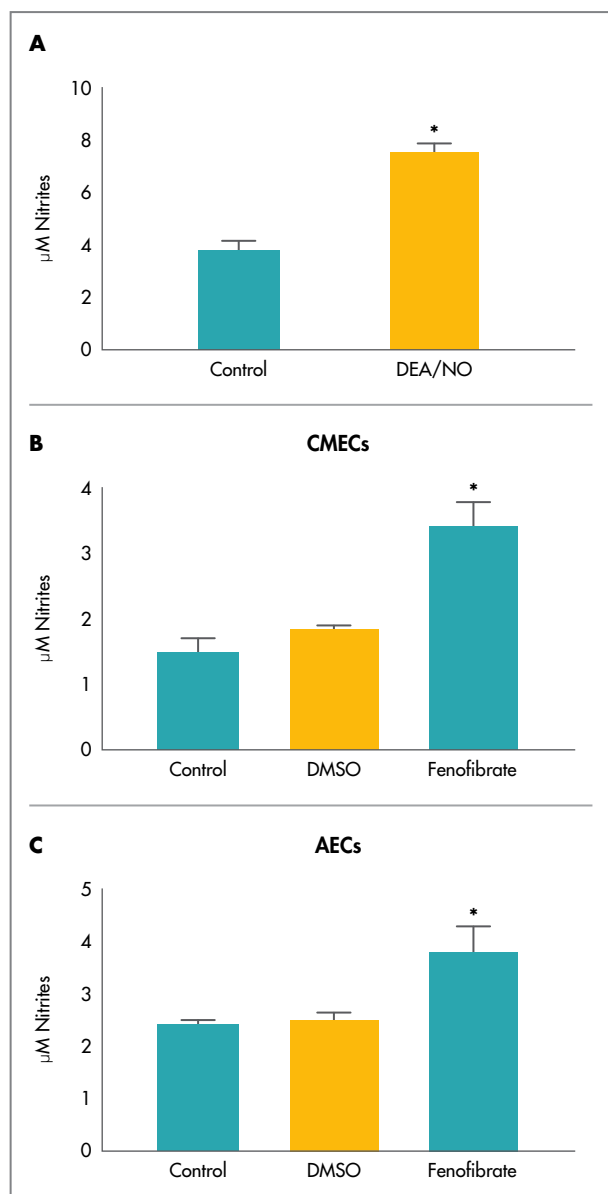
DAF-2/DA fluorescence measurements indicated that TNF-alpha treatment (20ng/ml; 24 hours) resulted in a 17% reduction in NO-production ( $p < 0.05$  vs. control) (Figure 4a), which was associated with a 2.9-fold increase in the fluorescence intensity of the mitochondrial ROS probe, MitoSox<sup>TM</sup> ( $p < 0.05$  vs. control) (Figure 4b). Furthermore, there was a 39% increase in the non-viable (propidium iodide-staining) cells in the TNF-alpha treatment group compared to control ( $p < 0.05$ ) (Figure 4c). Taken together, the aforementioned findings are highly suggestive of the induction of endothelial dysfunction in the TNF-alpha treated CMECs.

Fenofibrate pre-treatment (50 $\mu$ M) prevented the TNF-alpha-induced reduction in NO-production; in fact, NO measurements increased by 40% compared to TNF-alpha only treatment ( $p < 0.05$ ) (Figure 4a). In addition, there was a marked 2.5-fold reduction in mitochondrial ROS levels in the fenofibrate pre-treatment group compared to TNF-alpha only treatment ( $p < 0.05$ ) (Figure 4b). Finally, fenofibrate pre-treatment prevented the loss of cell viability observed in the TNF-alpha only treatment group ( $p < 0.05$ ) (Figure 4c).

### Fenofibrate pre-treatment increases eNOS (Ser 1177) and AMPK phosphorylation and prevents TNF-alpha-induced expression of p22PHOX and I $\kappa$ B $\alpha$

TNF-alpha treatment was associated with a ~45% reduction in eNOS expression compared to control, which was not affected by fenofibrate pre-treatment (Figures 5a, b). However, activated eNOS (phospho eNOS Ser 1177) levels increased by ~47% in the fenofibrate pre-treatment group compared to TNF-alpha treatment alone, and a ~41% increase was observed in the phosphorylated / total eNOS ratio of the fenofibrate pre-treatment group compared to control (Figures 5a, c, d). Although total AMPK expression was not altered in any of the groups, activated AMPK (phospho AMPK Thr 172) levels increased by ~49% in the fenofibrate pre-treatment group compared to TNF-alpha treatment alone (Figures 5a, f). Akt expression, activation and phosphorylated/total Akt ratio were not affected by any of the treatment protocols (Figures 5a, h).

Nitrotyrosine levels were not altered in any of the groups compared to control (Supplementary Figure S1). Fenofibrate pre-treatment reduced the expression of the NADPH-oxidase subunit p22PHOX by ~50% compared to TNF-alpha treat-



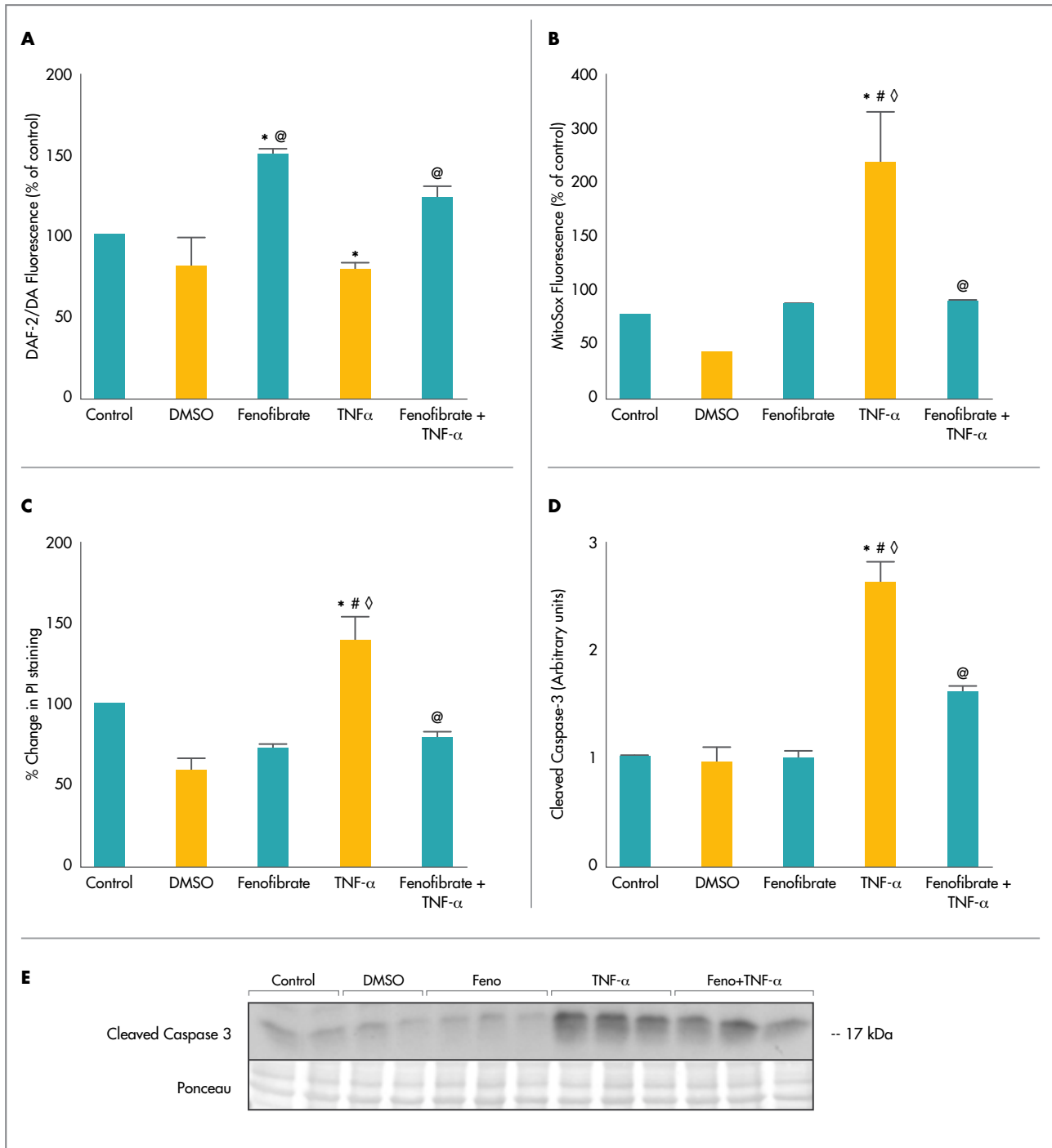
**FIGURE 3: Concentration nitrites as measured by the Griess assay in CMECs and AECs.**

A. DEA/NO (positive control) increased nitrite concentration in the supernatant;  $n = 7 - 8$  per group.

B. Concentration nitrites released into supernatant increased after 1 hour treatment with 50 $\mu$ M fenofibrate in CMECs;  $n = 8 - 13$ /group.

C. Concentration nitrites released into supernatant increased after 1 hour treatment with 50 $\mu$ M fenofibrate in AECs;  $n = 8 - 11$ /group.

\* =  $p < 0.05$  vs. control.



**FIGURE 4: Effects of pre-treatment with fenofibrate (50 $\mu$ M) administered one hour prior to TNF-alpha treatment (20ng/ml; 24 hours) on NO production, mitochondrial ROS generation and cell viability in CMECs.**

A. Intracellular NO production increased in the fenofibrate treatment group and decreased in TNF-alpha treatment group compared to control. Fenofibrate pre-treatment increased intracellular NO production compared to TNF-alpha treatment only. NO production was measured by DAF - 2/DA fluorescence and data expressed as % of control (control adjusted to 100%); n=4 - 11/group.

B. Mitochondrial ROS production increased in the TNF-alpha treatment group compared to control, and the increase was prevented by fenofibrate pre-treatment. Mitochondrial ROS production was measured by MitoSox™ Red fluorescence and data expressed as % of control (control adjusted to 100%); n=3 - 4/group.

C. TNF-alpha treatment increased the % non-viable cells compared to control and the increase was prevented by fenofibrate pre-treatment. Data expressed as % change in propidium iodide staining cells from control (control adjusted to 100%).

D. TNF-alpha treatment increased the levels of cleaved caspase-3 compared to control, and fenofibrate pre-treatment prevented the increase.

E. Representative Western blot of cleaved caspase-3; n=8 -15/group.

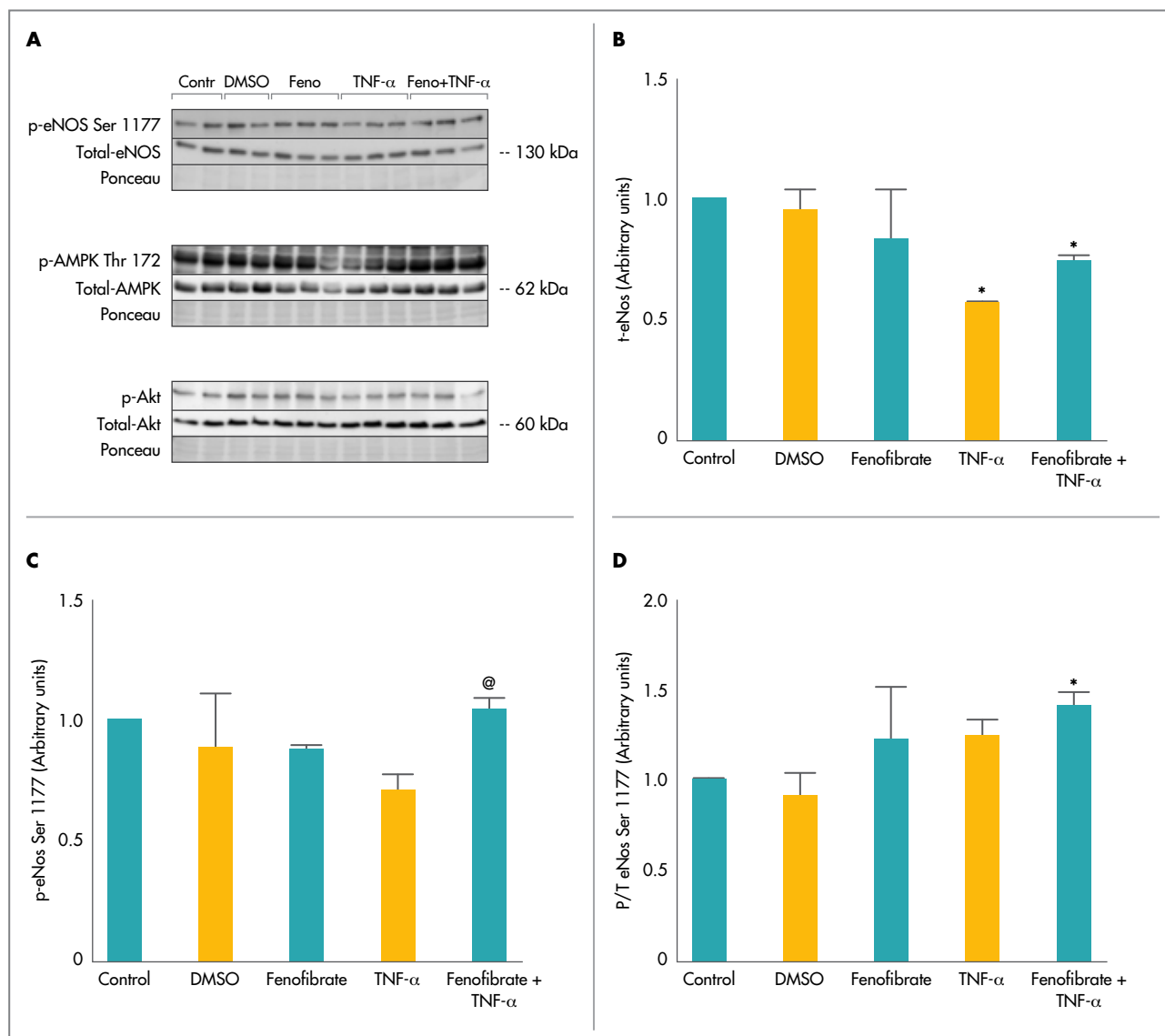
\* = p<0.05 vs. control, @ = p<0.05 vs. TNF- $\alpha$ , # = p<0.05 vs. DMSO, ◇ = p<0.05 vs. fenofibrate. Feno = Fenofibrate, Feno+TNF- $\alpha$  = Fenofibrate pre-treatment.

ment alone (Figures 6a, b). TNF-alpha treatment resulted in the activation of the NF-KB pathway as demonstrated by a decrease of ~36% in the expression of IκBα; however, fenofibrate pre-treatment failed to prevent the reduction in IκBα (Figures 6a, c). TNF-alpha treatment increased the expression of cleaved caspase-3 levels by ~2.6-fold compared to control, further signifying the induction of endothelial dysfunction, whilst fenofibrate pre-treatment prevented the increase in cleaved caspase-3 levels (Figures 4d, e).

## DISCUSSION

In the present study, we demonstrated that fenofibrate protects endothelial cells against the harmful effects of TNF-alpha, and that the protection is associated with the up-regulation of eNOS-NO biosynthesis, reduced ROS production, and prevention of cell death.

In the baseline experiments, healthy endothelial cells responded to short term fenofibrate treatment by a marked increase in NO production. This observation was further supported by a



**FIGURE 5.1: Effects of pre-treatment with fenofibrate (50μM) administered one hour prior to TNF-alpha treatment (20ng/ml; 24 hours) on the expression and phosphorylation of eNOS, AMPK and Akt in CMECs.**

A. Representative western blots of phosphorylated eNOS (ser 1177), total eNOS, phosphorylated AMPK (Thr 172), total AMPK, phosphorylated Akt (Ser 473), and total Akt.

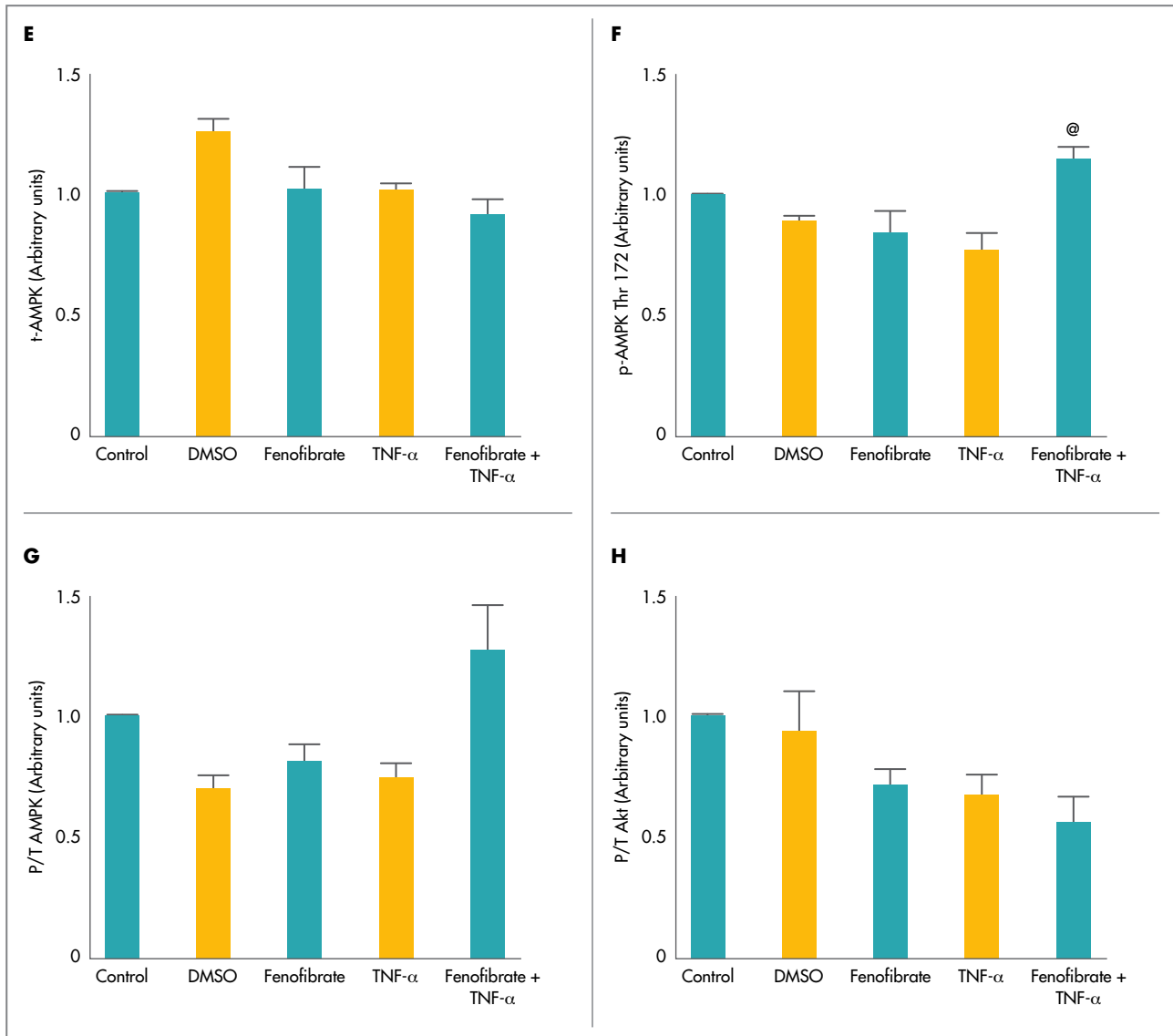
B. Total eNOS expression decreased in TNF-alpha only and fenofibrate pre-treatment groups compared to control.

C. Phosphorylated eNOS levels increased in fenofibrate pre-treatment vs. TNF-alpha only group.

D. Phosphorylated/total eNOS ratio increased in fenofibrate pre-treatment vs. control.

\* =  $p < 0.05$  vs. Control. @ =  $p < 0.05$  vs. TNF-alpha.  $n = 3$ /group. Fenofibrate, Fenofibrate + TNF-α = fenofibrate pretreatment.





**FIGURE 5.2: Effects of pre-treatment with fenofibrate (50µM) administered one hour prior to TNF-alpha treatment (20ng/ml; 24 hours) on the expression and phosphorylation of eNOS, AMPK and Akt in CMECs.**

E. Total AMPK expression was not affected by any of the treatment groups.

F. Phosphorylated AMPK levels increased in the fenofibrate pre-treatment group vs. TNF-alpha treatment only.

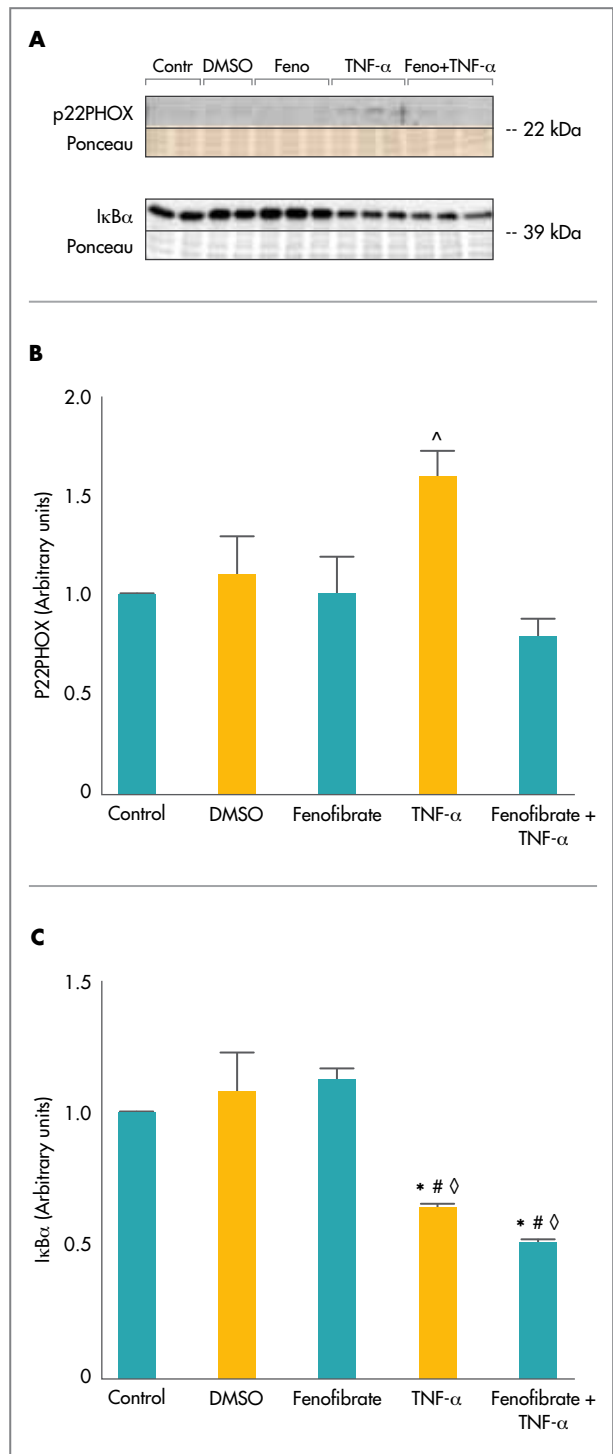
G. Phosphorylated/total AMPK ratio was not affected by any of the treatment groups.

H. Phosphorylated/total Akt ratio was not affected by any of the treatment groups.

\* =  $p < 0.05$  vs. Control. @ =  $p < 0.05$  vs. TNF-alpha.  $n = 3$ /group.

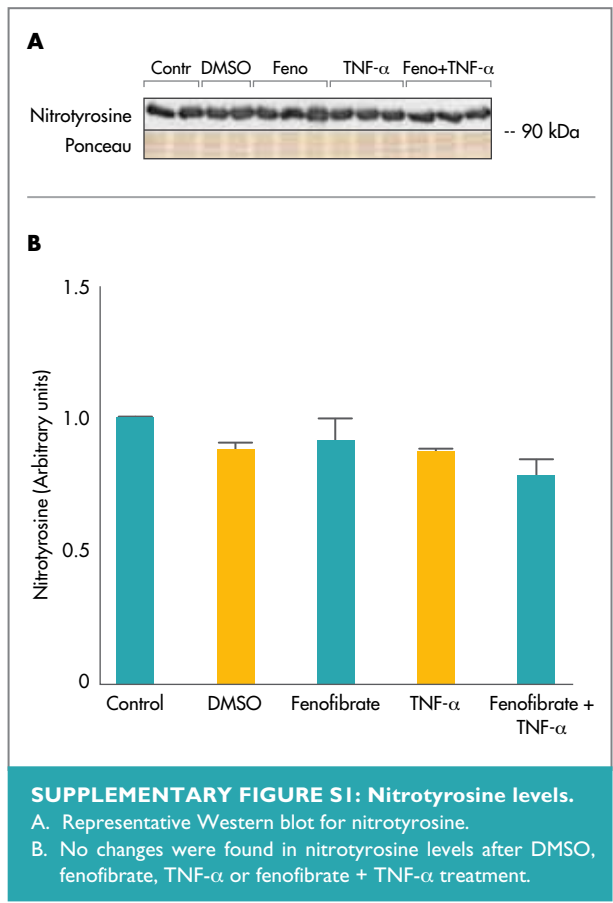
modest, but significant eNOS-dependent pro-vasodilatory response to fenofibrate administration in rat aortic rings. The ability of fenofibrate to increase NO production in endothelial cells has previously been demonstrated. For example, in cultured human umbilical vein endothelial cells (HUVECs), fenofibrate (30µM; 10 minutes) treatment resulted in a ~2-fold increase in NO production,<sup>(37)</sup> in line with the increases observed in our study. Similarly, a 2-fold increase in NO production was observed in human glomerular microvascular endothelial cells treated with fenofibrate (100µM) for 1 hour.<sup>(9)</sup> In bovine aortic endothelial cells, fenofibrate (50µM) was shown

to increase eNOS expression and activity after 48 hours treatment; however, the authors did not measure NO production.<sup>(38)</sup> The general consensus in the literature that fenofibrate up-regulates NO biosynthesis in vascular endothelial cells has potentially important implications, as NO is associated with beneficial effects in the cardiovascular system via, among others, anti-inflammatory, anti-apoptotic and anti-necrotic mechanisms.<sup>(39)</sup> In our hands, fenofibrate treatment affected neither baseline superoxide production, nor intracellular levels of the harmful radical peroxynitrite compared to control conditions, demonstrating that the large increases in NO



**FIGURE 6: Effects of pre-treatment with fenofibrate (50µM) administered one hour prior to TNF-alpha treatment (20ng/ml; 24 hours) on the expression of p22PHOX, IκBα and cleaved caspase-3.**  
 A. Representative western blots of p22PHOX and IκBα.  
 B. Fenofibrate pre-treatment decreased the expression of p22PHOX compared to TNF-alpha only.  
 C. The expression of IκBα was decreased compared to control in the TNF-alpha only and fenofibrate pre-treatment groups.

\* = p<0.05 vs. Control. # = p<0.05 vs. DMSO. ◊ = p<0.05 vs. Fenofibrate, @ = p<0.05 vs. TNF-alpha. ^ = p<0.05 vs. Fenofibrate + TNF-alpha, n = 3/group. Fenofibrate = fenofibrate, Fenofibrate + TNF-α = fenofibrate pre-treatment.



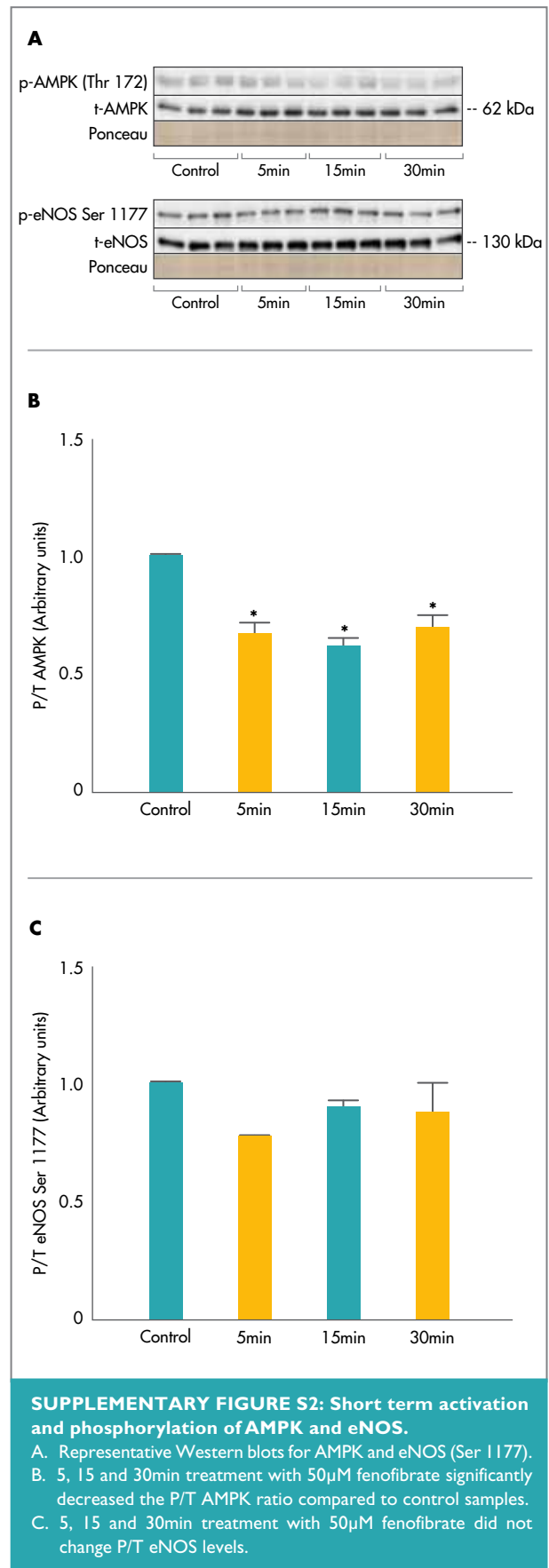
**SUPPLEMENTARY FIGURE S1: Nitrotyrosine levels.**  
 A. Representative Western blot for nitrotyrosine.  
 B. No changes were found in nitrotyrosine levels after DMSO, fenofibrate, TNF-α or fenofibrate + TNF-α treatment.

n = 3/group.  
 production were not associated with oxidative or nitrosative stress. Cell viability remained unaffected compared to untreated control conditions.

Following the baseline studies in healthy CMECs, we investigated whether the effects of fenofibrate would translate into protection in a model of dysfunctional endothelial cells induced by a harmful inflammatory stimulus, with regards to NO production, ROS generation and cell viability. To our knowledge, the endothelioprotective properties of fenofibrate have not previously been demonstrated in this context. Previous data from our laboratory showed that 24 hours treatment of CMECs with TNF-alpha at 20ng/ml resulted in endothelial dysfunction as determined by reduced NO production, eNOS down-regulation and increased oxidative stress.<sup>(40)</sup> The same concentration and treatment period were used in the present study, and endothelial dysfunction was demonstrated by reduced eNOS protein expression associated with decreased NO levels, increased mitochondrial ROS generation, decreased cell viability and increased apoptosis. Furthermore, the pro-inflammatory effects of TNF-alpha were confirmed by increased NF-KB activity. In addition to our own previous findings,<sup>(40)</sup> others have also shown that TNF-α down-regulates eNOS and/or reduces NO production in various models of endothelial

cells.<sup>(41-43)</sup> Furthermore, the induction of ROS generation by TNF-alpha,<sup>(44,45)</sup> as well as its pro-apoptotic effects<sup>(47,47)</sup> have extensively been described.

We have demonstrated that fenofibrate pre-treatment protected the endothelial cells against the harmful effects of TNF-alpha with regards to NO production, ROS generation and cell viability. The protection was characterised by the prevention of a decrease in NO production, the prevention of an increase in mitochondrial ROS production and p22PHOX expression, the prevention of a loss of cell viability, and the prevention of increases in cleaved caspase-3 levels. Pre-treatment of CMECs with fenofibrate resulted in increased activated eNOS (p-eNOS Ser 1177) levels compared to cells treated with TNF-alpha only, as well as a significantly greater p-eNOS Ser 1177 / total eNOS ratio compared to untreated control. The up-regulation of eNOS activation was associated with a ~40% increase in NO generation compared to TNF-alpha only. Indeed, many of the pleiotropic effects of fenofibrate on vascular endothelium have been attributed to eNOS phosphorylation and activation;<sup>(9,37,38)</sup> however, most studies have not investigated fenofibrate-induced effects on the eNOS-NO biosynthesis pathway in the context of injured or dysfunctional endothelial cells. AMPK and Akt are regarded as two of the most important upstream regulators of eNOS activation via Ser 1177 phosphorylation.<sup>(48)</sup> In the present study, fenofibrate pretreatment increased activated AMPK (phospho AMPK Thr 172) levels compared to TNF-alpha only treatment, which corresponds with the increased activation of eNOS. In this regard, fenofibrate has previously been associated with increased phosphorylation of AMPK (Thr 172) in a variety of endothelial cell types;<sup>(9,37,49,50)</sup> however, none of these studies were conducted in the context of TNF-alpha induced injury. Tomizawa, et al. (2011) showed that 100µM fenofibrate activates AMPK and Akt in human glomerular microvascular endothelial cells with resultant activation of eNOS (Ser 1177) within 1 hour of treatment. Similarly, Murakami, et al. (2006) demonstrated that fenofibrate activates AMPK and eNOS (Ser 1177) in HUVECs. These findings were observed at a concentration of 30µM at 10 minutes. Kim, et al. (2007) showed fenofibrate protects endothelial cells against apoptosis via a PPARα independent, AMPK dependent activation. Although the latter study used a similar concentration of fenofibrate (50µM) compared to ours, it was performed in retinal endothelial cells and treatment lasted for 1 hour. From the literature, it is clear that eNOS and AMPK can be activated within minutes of stimulation.<sup>(37)</sup> Although we did perform experiments to investigate the short term effects of fenofibrate on eNOS and AMPK activation (5, 15 and 30 minutes), no evidence of activation by phosphorylation was detected (Supplementary, Figure S2). None of the treatment protocols in the present study exerted any effects on Akt expression or activation. It therefore seems that upstream Akt played no role in the phosphorylation of eNOS in our hands. The literature appears to be contradictory regarding fenofibrate's effects on Akt; whereas some studies showed an association with increased phosphorylation of Akt (Thr 308) in human glomerular



\* = p<0.05 vs. control, n = 3/group.

microvascular endothelial cells<sup>(9)</sup> and Akt (Ser 473) in hepatocytes,<sup>(51)</sup> others demonstrated inhibition of the Akt pathway in HUVECs,<sup>(52)</sup> and hepatocytes.<sup>(53)</sup>

The induction of 2 oxidative stress markers by TNF-alpha in the CMECs (p22PHOX and mitochondrial ROS levels) was prevented by fenofibrate pretreatment. Fenofibrate's antioxidant properties have previously been demonstrated in an in vivo study by reducing superoxide anion levels and p22PHOX expression in a model of nicotine-treated rat aortic tissue;<sup>(54,55)</sup> however the authors did not investigate the effects in endothelial cells. Although fenofibrate pretreatment did not alter TNF-alpha-induced activation of NF-KB in our hands, it was associated with a marked improvement in cell viability and reduced caspase-3 mediated apoptosis. NO appears to have paradoxical effects on apoptosis.<sup>(56)</sup> NO can inhibit caspase-3 by s-nitrosation,<sup>(57)</sup> and it is possible that fenofibrate resulted in reduced caspase-3 mediated apoptosis due to increased levels of NO. Although anti-apoptotic effects of fenofibrate have previously been demonstrated in human retinal endothelial cells subjected to serum deprivation,<sup>(49)</sup> and in HUVECs exposed to high glucose,<sup>(10)</sup> we could not find any studies investigating the anti-apoptotic effects of fenofibrate in the context of TNF-alpha induced endothelial injury. Repeating pre-treatment studies in the presence of a NOS-inhibitor would have added great value to the study, and the lack thereof is acknowledged as a shortcoming.

In conclusion, the present study provides novel data demonstrating that fenofibrate pretreatment protects adult rat endothelial cells against the harmful, pro-endothelial dysfunction effects of TNF-alpha with regards to NO and ROS production, and cell viability parameters. The findings are important in view of the fact that therapeutic interventions may prevent endothelial dysfunction. Inflammation has emerged as a pivotal role-player in the pathophysiology of arterial diseases (reviewed in 15) and therapies aimed at preventing this condition should be further researched. Although our data suggest that fenofibrate shows potential as a possible future therapeutic option in the preventative treatment of TNF-alpha-induced endothelial dysfunction, more studies are needed to further explore and validate these promising observations.

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