Tumour necrosis factor-alpha participates on the endothelin-1/nitric oxide imbalance in small arteries from obese patients: role of perivascular adipose tissue

Agostino Virdis[1]*, Emiliano Duranti[1], Chiara Rossi[1], Umberto Dell'Agnello[1], Eleonora Santini[1], Marco Anselmino[2], Massimo Chiarugi[3], Stefano Taddei[1], and Anna Solini[1]

[1]Department of Clinical and Experimental Medicine, University of Pisa, Via Roma, 67, 56100 Pisa, Italy; [2]Surgery Unit IV, University Hospital of Pisa, Pisa, Italy; and [3]Department of Surgery, Medical, Molecular, and Critical Area Pathology, University of Pisa, Pisa, Italy

Aims	We assessed the impact of vascular and perivascular tumour necrosis factor-alpha (TNF-α) on the endothelin (ET)-1/ nitric oxide (NO) system and the molecular pathways involved in small arteries from visceral fat of obese patients (Obese) and Controls.
Methods and results	Isolated small arteries from 16 Obese and 14 Controls were evaluated on a pressurized micromyograph. Endogenous ET-1 activity was assessed by the ET_A blocker BQ-123. TNF-α and NO were tested by anti-TNF-α infliximab (IFX) and N^{ω}-nitro-L-arginine methylester (L-NAME). Gene and protein expression of TNF-α, ET-1, ET_A, and ET_B receptors were determined by RT-PCR and IHC on arterial wall and in isolated adipocytes. Obese showed a blunted L-NAME-induced vasoconstriction, which was potentiated by IFX, and an increased relaxation to BQ-123, unaffected by L-NAME but attenuated by IFX. Perivascular adipose tissue (PVAT) removal reversed these effects. Obese showed intravascular superoxide excess, which was decreased by apocynin (NAD(P)H oxidase inhibitor), L-NAME, and BQ-123 incubations, and abolished by IFX. An increased vascular expression of ET-1, ET_A, and ET_B receptors, and higher vascular/ perivascular TNF-α and TNF-α receptor expression were also detected. The arterial expression and phosphorylation of c-Jun N-terminal kinase (JNK) were higher in Obese vs. Controls, and downregulated by IFX.
Conclusions	In small arteries of Obese, PVAT-derived TNF-α excess, and an increased vascular expression of ET-1 and ET_A receptor, contribute to the ET-1/NO system imbalance, by impairing tonic NO release. Reactive oxygen species excess, via NAD(P)H oxidase activation, induces the endothelial nitric oxide synthase uncoupling, which in turn generates superoxide and impairs NO production. The up-regulated JNK pathway represents a crucial molecular signalling involved in this process.
Keywords	Perivascular adipose tissue • TNF-α • Endothelin-1 • Nitric oxide • Small vessels

Introduction

In physiological conditions, vascular homeostasis is guaranteed by the opposite action of endothelium-derived relaxing and contracting factors, mainly nitric oxide (NO) and endothelin (ET)-1. Endothelin-1 is a vasoconstrictor peptide which acts by binding to two receptor subtypes, ET_A and ET_B, located on vascular smooth muscle cells and able to mediate the vast majority of ET-1 effects.[1] ET_B receptors are also found on endothelial cells, where their activation mainly results in NO-mediated vasodilation; in turn, NO counterbalances the contracting effect of ET-1.[1,2]

Obesity is characterized by endothelial dysfunction.[3–7] Such alteration includes a vascular ET-1 /NO imbalance, in favour of an abnormal activation of the endogenous ET-1 system. Thus, an increased

---

ET-1-mediated vasoconstrictor tone, simultaneously with a reduced tonic NO release, is demonstrated in peripheral microcirculation of obese patients.[8,9]

Obesity is also a condition of chronic low-grade inflammation secondary to an abnormal production of proinflammatory cytokines, including tumour necrosis factor-alpha (TNF-α), which may negatively influence vascular reactivity.[10,11] In this picture, a relevant role is played by perivascular adipose tissue (PVAT), which *per se* reduces NO availability.[12] More recently, it has been proposed that TNF-α, represented either in adipose tissue and in the vascular wall of obese patients, stimulates the reactive oxygen species (ROS) generation, mainly through NAD(P)H oxidase activation, which in turn reduces the endothelial agonist-evoked NO availability.[6,13] At present, the possibility that local inflammation might also interfere with the ET-1-mediated vascular tone in obesity is still unknown. It is documented that ROS induce ET-1 expression, and at the same time, ET-1 appears to stimulate ROS production.[14,15] Based on this knowledge, oxidant excess can be hypothesized as a mechanism whereby TNF-α interferes with the ET-1/NO system. Therefore, the aim of the present study was to evaluate whether TNF-α contributes to the vasoconstriction induced by endogenous ET-1 in small arteries isolated from the visceral abdominal fat of patients with severe obesity, and whether this effect might be indirectly mediated by a modulation of tonic NO release. The possibility that endothelial NO synthase (eNOS) uncoupling might occur, to impair tonic NO release as well as a contributor of ROS generation, and its relationship with TNF-α was also assessed. Finally, we investigated the specific role of PVAT on the ET-1/NO system and the intracellular molecular pathways throughout TNF-α modulates ET-1 effects.

Methods

Study population

The study included 16 severe obese patients (Obese) and 14 matched non-obese control subjects (Controls). The protocol was approved by the local Ethical Committee, and an informed written consent was obtained from each participant. For inclusion criteria of the participants, see the online-only Data Supplement.

Preparation and mounting of small arteries

All participants underwent a biopsy of visceral fat, taken during surgical laparoscopic procedure. Small arteries (150–300 μm) were isolated immediately and mounted in a pressurized myograph, as previously described.[16,17] From each artery, one segment was cleaned of PVAT (PVAT$-$), whereas on the adjacent segment PVAT was left intact (PVAT$+$). Experiments performed with vasoconstrictor compounds were conducted in quiescent vessels. In contrast, in those experiments involving relaxing compounds, vessels were pre-contracted with NA (1 μM), which elicited similar contractions among the experimental groups (data not shown). The concentrations and times of infusions of the compounds utilized were chosen according to preliminary dose-titration experiments, to establish the threshold dose able to elicit a maximal and stable vasoactive effect (Supplementary material online, *Figure S1*). See also online-only Data Supplement.

Assessment of the effect of tumour necrosis factor-α on tonic nitric oxide release

The impact of TNF-α on vascular tone was assessed by the anti-TNF-α monoclonal antibody infliximab (IFX), while the tonic NO release was ascertained by the NO synthase inhibitor N^{ω}-nitro-L-arginine methylester (L-NAME). Thus, quiescent small vessels PVAT$+$ from Obese and Controls were incubated for 30 min with L-NAME (100 μM), IFX (100 μM), and IFX plus L-NAME. The same protocol was repeated in PVAT$-$ vessels.

Nitrite levels

PVAT$+$ and PVAT$-$ small vessels from Obese and Controls were incubated in Dulbecco's modified Eagle's medium, without or with IFX. Basal NO production was measured using Nitrate/Nitrite Colorimetric Assay Kit (Cayman) by Griess reaction as previously described.[18] (See Supplementary material online.)

Assessment of the effect of tumour necrosis factor-α on endothelin-1/nitric oxide system

Endogenous ET-1-elicited vasoconstriction was investigated by the selective ET_A receptor antagonist BQ-123. To ascertain the effect of TNF-α on the balance between the endogenous ET-1-mediated vasoconstrictor tone and the NO release (ET-1/NO system), pre-contracted PVAT$+$ and PVAT$-$ small vessels from Obese and Controls were incubated for 30′ with IFX (100 μM), BQ-123 (1 μM), or both, without or with L-NAME (100 μM). Finally, as control experiments, in pre-contracted PVAT$+$ vessels from Obese and Controls, the endothelium-independent relaxation and the vasoconstrictor sensitivity to ET-1 were assessed by cumulative concentrations of sodium nitroprusside (0.01–100 μM) and exogenous ET-1 (10 nM–1 μM), respectively.

Detection of vascular superoxide anion generation

The *in situ* production of superoxide anion was measured by the fluorescent dye dihydroethidium (DHE; Sigma), as previously described.[6] Each segment was analysed simultaneously after incubation with apocynin (100 μM), L-NAME (100 μM), IFX (100 μM), BQ-123 (100 μM), or Krebs solution. (See Supplementary material online.)

Adipocyte isolation

Mature adipocytes were isolated from PVAT according to Rodbell.[19] Briefly, tissue samples were digested with collagenase type II (C6885, Sigma) at 37°C in HANKS' balanced salt solution for 1 h. Following centrifugation at 1900 rpm, the superficial oil was removed, the suspension was filtered (210-μm filter), and the suspended cells were repeatedly washed with PBS before storing them at -80°C.

Analysis of gene expression

Total RNA was extracted with the RNasy lipid tissue mini kit. One microgram RNA was reverse-transcribed in a 20 μl reaction tube using High-Capacity cDNA Reverse transcription kit. Reactions were run in a MJ mini thermocycler at 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min. Real-time PCR was performed in triplicate on an Eco-Real Time instrument following a standard protocol (40 cycles of denaturation at 95°C for 15 s followed by annealing and extension at 60°C for 1 min). Amplifications were normalized by GAPDH, and quantitation of gene expression was performed using the $\Delta\Delta C_T$ calculation, where C_T is the threshold cycle. The amount of the target gene, normalized to

GAPDH and relative to the calibrator (total RNA from an healthy control), is given as $2^{-\Delta\Delta CT}$.

Immunostaining

In each subject, one of arteries isolated for the pharmacological study was processed for immunostaining. After a short step of fixation (1 h at room temperature) with 4% paraformaldehyde in phosphate buffer pH 7.2, the artery was cryoprotected overnight at 4°C in 20% sucrose; the next day, it was included in TissueTek and stored a −20°C. Transverse 12-μm thick sections were cut with a cryostat microtome and serially mounted on SuperfrostPlus slides. After rinsing three times (10 min each) with PBS pH 7.4, arterial sections were treated with 8% BSA in PBS for 1 h, to block non-specific binding. After reacting overnight at 4°C with specific primary antibodies (see Supplementary material online), sections were incubated with 488-conjugated donkey anti rabbit antibody (1:400 in PBST). After covering the slides using an antifade mounting medium, selected images were acquired with a laser scanning confocal microscope.

Western blot analysis

Refer to Supplementary material online.

Drugs, reagents, and solutions

Refer to Supplementary material online.

Statistics

Results were calculated as maximal percentage increments or decrements of lumen diameter from baseline, in quiescent or NA-mediated pre-contracted vessels, when appropriate. Data were presented as mean ± SE, and analysed by repeated measures ANOVA, followed by the Student−Newman−Keuls test, by one-way ANOVA using the Tukey test for multiple comparisons, or by two-sided unpaired Student's *t*-test, where appropriate. A value of $P < 0.05$ was considered statistically significant. All analyses were performed using Prism software (GraphPad Software Inc, version 5.0).

Results

Clinical characteristics of the study populations are reported in *Table 1*. Obese had higher BMI and waist circumference values when compared with Controls, as well as systolic and diastolic blood pressure values, even within the normal range. Fasting insulin, HOMA-IR, and plasma TNF-α values were also higher in Obese than in Controls.

Effect of tumour necrosis factor-α on tonic nitric oxide release in quiescent vessels: role of perivascular adipose tissue

In quiescent PVAT+ vessels, IFX did not change significantly resting tone, either in Controls or in Obese. In Controls, L-NAME induced a significant vasoconstriction, which was resistant to IFX (*Figure 1*). In contrast, in vessels from Obese, the L-NAME-mediated vasoconstriction was attenuated compared with Controls and increased by IFX incubation, resulting no longer different from that in Controls (*Figure 1*).

Perivascular adipose tissue removal had no influence on the lacking effect of IFX in vascular resting tone, either in Controls or Obese. In PVAT− vessels from Controls, L-NAME-induced vasoconstriction was significantly attenuated when compared with corresponding

Table I Clinical characteristics of the two study groups

Parameter	Obese (*n* = 16)	Controls (*n* = 14)	*P*-value
Age	45.3 ± 6.2	46.1 ± 7.0	0.73
Male/female	6/10	6/8	0.77
Body mass index (kg/m^2)	44.5 ± 4.7*	25.6 ± 4	<0.0001
Waist circumference (cm)	129.9 ± 11.9*	80.7 ± 8.3	<0.0001
Systolic blood pressure	137 ± 4*	133 ± 5	<0.05
Diastolic blood pressure	84 ± 4*	78 ± 4	<0.05
Total cholesterol (mg/dL)	204 ± 18	194 ± 14	0.10
Triglycerides (mg/dL)	150 ± 14	138 ± 19	0.07
Fasting glucose (mg/dL)	90 ± 9	85 ± 10	0.13
Fasting insulin (μU/mL)	12.6 ± 2.4*	9.1 ± 2.2	<0.05
HOMA-IR	2.8 ± 0.7*	1.9 ± 0.5	<0.05
TNF-α (pg/mL)	6.95 ± 2.46*	2.72 ± 0.93	<0.05

PVAT+ vessels, and not affected by concomitant IFX infusion (*Figure 1*). In vessels from Obese, PVAT removal potentiated the L-NAME-mediated vasoconstriction ($P < 0.01$ vs. PVAT+ vessels). The simultaneous IFX incubation still exerted a potentiating effect on L-NAME-induced contraction, but this effect was less marked ($P < 0.05$ vs. PVAT+) (*Figure 1*).

Effect of tumour necrosis factor-α on nitrite levels

At baseline, culture medium of incubated small arteries from Obese showed a significantly blunted production of nitrite levels, compared with Controls. Infliximab, while not affecting nitrite production in Controls, significantly increased NO production in Obese. Perivascular adipose tissue removal increased nitrite production among Obese, an effect still enhanced by IFX incubation (see Supplementary material online, *Figure S2*).

Effect of tumour necrosis factor-α on endothelin-1/nitric oxide system in pre-contracted vessels: role of perivascular adipose tissue

In pre-contracted PVAT+ vessels from Controls, IFX induced a slight relaxation, an effect abolished by simultaneous L-NAME. BQ-123 induced a vascular relaxation, which was significantly blunted by L-NAME but not affected by IFX. The inhibitory effect of L-NAME on the ET$_A$ antagonist was independent of IFX co-incubation (*Figure 2A*).

In pre-contracted PVAT+ vessels from Obese, IFX elicited a marked relaxation with respect to Controls ($P < 0.001$), which
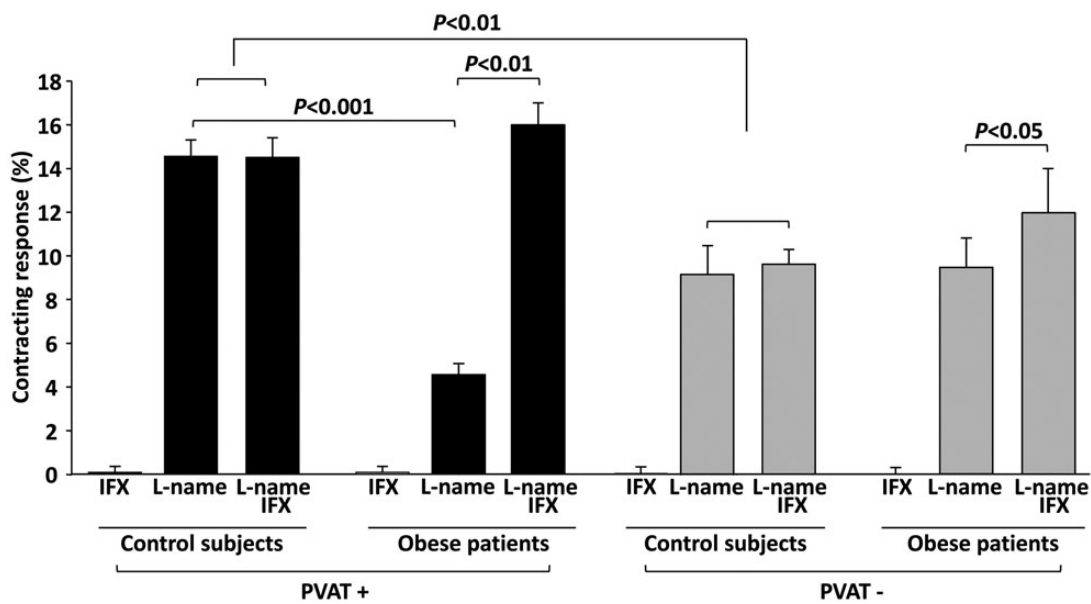
Figure 1 Per cent contracting response to infliximab, N^{ω}-nitro-L-arginine methylester, or both, in quiescent small vessels with perivascular adipose tissue or without perivascular adipose tissue from control subjects or obese patients. Each column represents the mean of seven experiments $\pm$ standard error of the mean and analysed by the one-way analysis of variance.
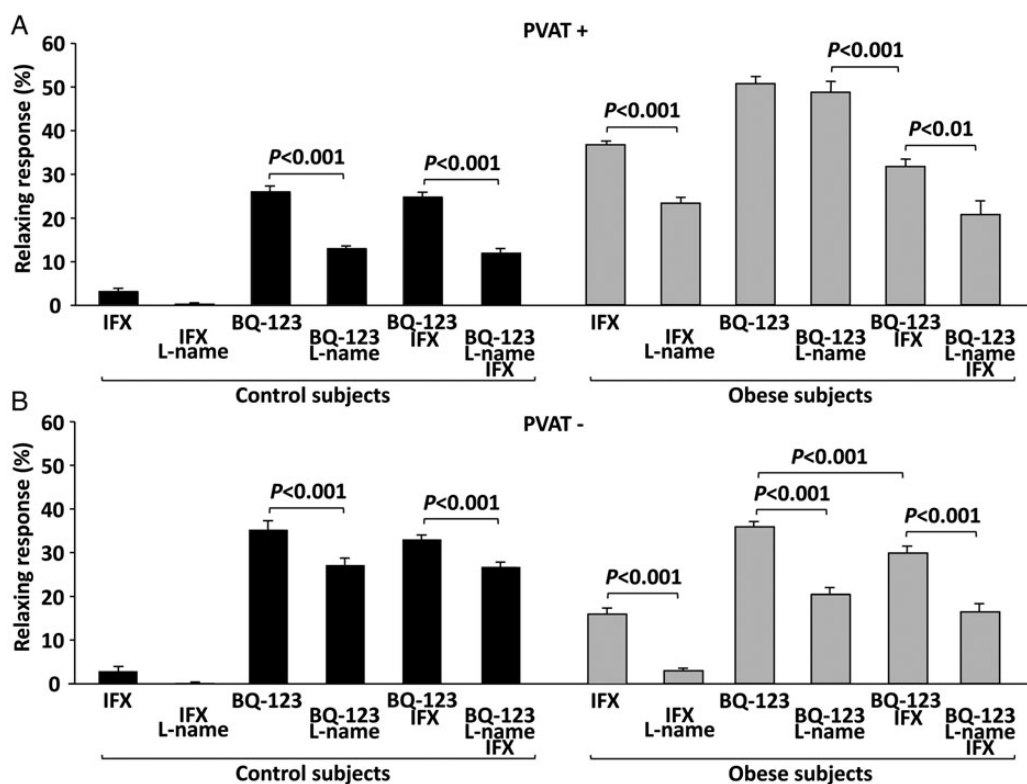

Figure 2 Per cent relaxing response to infliximab and BQ-123 $\pm$ N^{ω}-nitro-L-arginine methylester, infliximab or both, in pre-contracted small vessels with perivascular adipose tissue (*A*) or without perivascular adipose tissue (*B*) from control subjects or obese patients. Each column represents the mean of seven experiments $\pm$ standard error of the mean and analysed by the one-way analysis of variance.

was blunted by L-NAME. BQ-123 also resulted in a greater relaxation ($P < 0.001$ vs. Controls), which was resistant to L-NAME but significantly attenuated by IFX. Under simultaneous IFX and L-NAME incubations, the BQ-123 relaxation was dramatically attenuated (*Figure 2A*).

In PVAT− vessels from Controls, BQ-123 exerted a greater relaxation than that from PVAT+ segments ($P < 0.05$). In such vessels, while IFX was ineffective, the inhibition by L-NAME on the ET_A receptor antagonist was blunted compared with PVAT+ segments, an effect not modified by simultaneous L-NAME and IFX incubations (*Figure 2B*).

In PVAT− vessels from Obese, the IFX-mediated relaxation was attenuated ($P < 0.01$ vs. PVAT+) and greatly blunted by L-NAME. The BQ-123-mediated relaxation was also reduced ($P < 0.05$ vs. PVAT+). Relaxation to BQ-123 was still blunted by IFX, but this effect was less evident than that obtained in PVAT+ vessels ($P < 0.05$ vs. PVAT+). L-NAME incubation inhibited the relaxation by the ET_A receptor antagonist, an effect further exacerbated by the concomitant L-NAME and IFX incubations (*Figure 2B*). Maximal relaxation to sodium nitroprusside was similar in Obese ($94.5 \pm 1.1\%$) and Controls ($95.1 \pm 1.4\%$). Finally, vessels from Obese showed an increased vasoconstriction to exogenous ET-1 compared with Controls (see Supplementary material online, *Figure S3*).

Analysis of vascular superoxide anion generation

Dihydroethidium analysis revealed a dramatic increase in the superoxide anion production in vessels from Obese, compared with Controls. Such excess was decreased by apocynin, L-NAME, or their simultaneous incubation in a similar manner. BQ-123 incubation also decreased intravascular superoxide to a similar extent. Finally, IFX incubation totally abrogated superoxide generation (*Figure 3*).

Tumour necrosis factor-α, adiponectin, immune cells, monomeric and dimeric endothelial nitric oxide synthase determinations

Obese showed a significantly higher vascular TNF-α protein expression, coupled with an increased presence of TNF-α receptor 1 in the arterial wall (*Figure 4A*). To further assess the presence of this cytokine in the adipose tissue surrounding arteries, we also quantified TNF-α in PVAT, confirming the same trend observed in the arteries (*Figure 4B*). Adiponectin was less expressed in PVAT from Obese compared with Controls (*Figure 4C*). Moreover, we determined the degree of immune cell infiltration in the vascular wall, finding a significantly higher presence of CD68-positive cells in Obese (Supplementary material online, *Figure S4*). Finally, we evaluated monomeric and dimeric eNOS protein expression. While no difference was present in monomeric isoform, the dimeric isoform was more represented in Controls than Obese (Supplementary material online, *Figure S4*).

Immunostaining of endothelin-1, endothelin-A, and endothelin-B receptors

In order to provide an anatomic basis supporting the above-reported observations, we quantified ET-1 expression in arterial wall of Obese and Controls. A greater intravascular ET-1 presence was detected in Obese when compared with Controls (*Figure 5*). In addition, an enhanced expression of both ET_A and ET_B receptors in vessels from Obese was observed (*Figure 5*).

Intracellular signalling

We tested the arterial expression of c-Jun N-terminal kinase (JNK), p38 MAPK, and extracellular signal-regulated kinase (ERK), intracellular kinases involved in TNF-α signalling, and their response to IFX. Obese had a slightly higher JNK expression and phosphorylation, while p38 was reduced, either in the total and activated isoforms; ERK did not significantly differ between Obese and Controls (*Figure 6*). Interestingly, IFX was able to reduce JNK, p38, and ERK expression and phosphorylation much more in Obese, slightly affecting these pathway in arteries of lean subjects (*Figure 6*). As expected, total and phosphorylated eNOS was significantly attenuated in Obese (0.062 ± 0.041 vs. 0.038 ± 0.022 AU by densitometric analysis, $P < 0.05$ vs. Controls).

Discussion

The main novel finding of the present study is that IFX significantly potentiates the attenuated tonic NO release and, concomitantly, reduces the enhanced ET_A-dependent contracting activity in small vessels from Obese. These findings indicate that TNF-α directly interferes with the vascular ET-1/NO system, thus greatly contributing to the imbalance of the vascular homeostasis in obesity condition.

Role of tumour necrosis factor-α on vascular nitric oxide release

Small vessels from Obese showed an impaired tonic NO release, as previously demonstrated,[9] together with a blunted basal nitrite production. Accordingly, a reduced eNOS expression was detected in these vessels. Infliximab normalized the contracting response to L-NAME and increased the nitrite levels, thus indicating a major role of TNF-α in reducing NO activity in obesity. We recognize that detection of nitrite production, obtained in basal conditions, does not allow to discriminate between the eNOS and iNOS provenance. Perivascular adipose tissue removal enhanced the vasoconstriction by L-NAME, blunted the potentiating activity of IFX on L-NAME, and enhanced the nitrite production. An increased TNF-α expression was detected in isolated adipocytes from Obese. In conjunction, these findings highlight the role of PVAT as a major source of TNF-α; the presence of vascular TNF-α receptor-1 indicates the vasculature as the main target of such TNF-α excess. Our findings also confirm that local inflammation abolishes the anticontractile properties of PVAT.[12] Indeed, PVAT removal from control vessels did not affect the missing effect of IFX, but dramatically attenuated the contracting response to L-NAME, thus supporting the existence of a vasodilator factor released by PVAT in physiological conditions. Greenstein *et al.*[12] documented the ability of healthy PVAT to secrete adiponectin, which increases NO availability. We found a reduced adiponectin expression in PVAT from Obese, suggesting that its missing effect can contribute to the NO inhibition in obesity. Of note, in obesity PVAT removal did not completely abolish the favouring effect of IFX on L-NAME. This finding, together
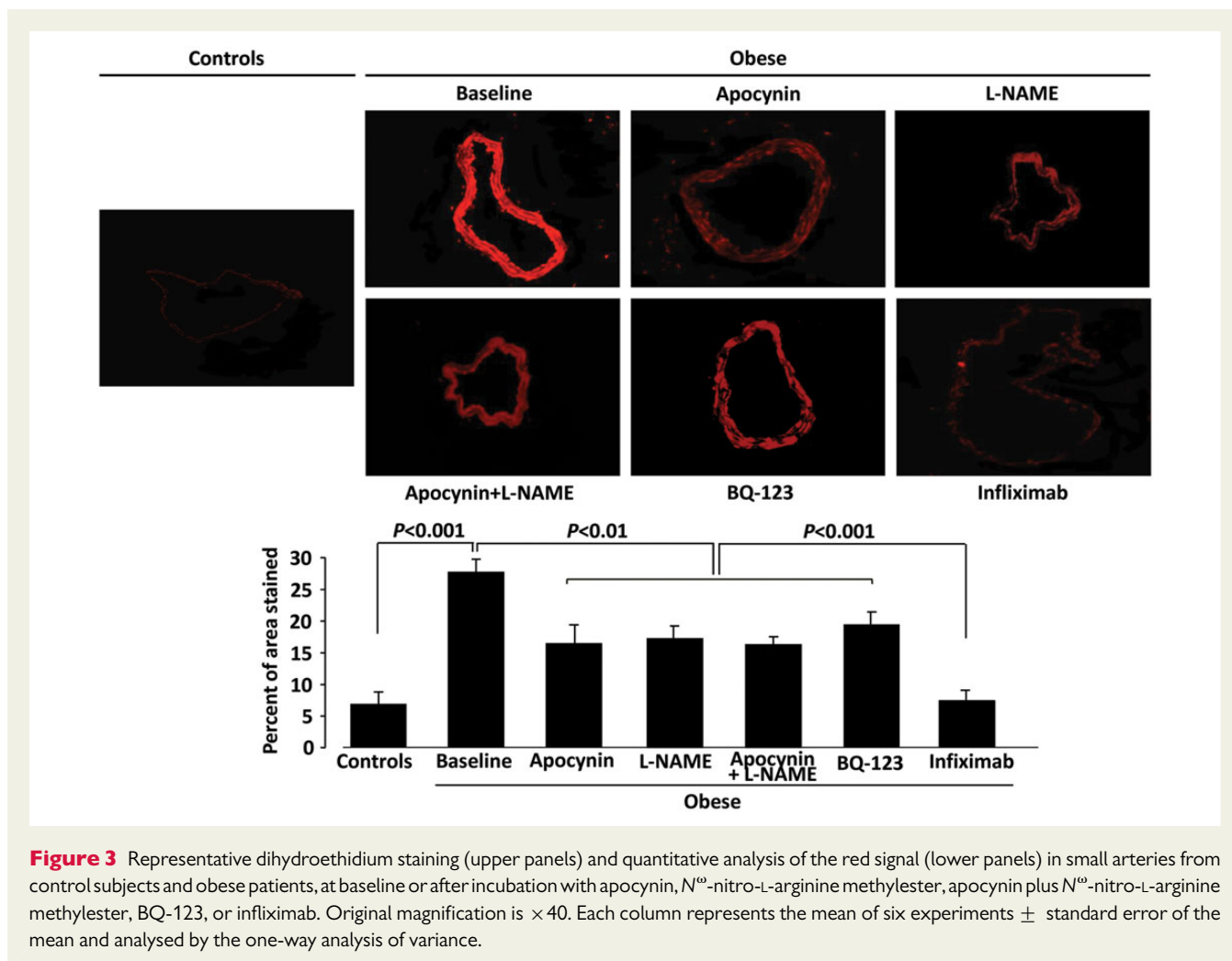
Figure 3 Representative dihydroethidium staining (upper panels) and quantitative analysis of the red signal (lower panels) in small arteries from control subjects and obese patients, at baseline or after incubation with apocynin, N^{ω}-nitro-L-arginine methylester, apocynin plus N^{ω}-nitro-L-arginine methylester, BQ-123, or infliximab. Original magnification is $\times 40$. Each column represents the mean of six experiments $\pm$ standard error of the mean and analysed by the one-way analysis of variance.

with the higher vascular TNF-α protein expression detected in Obese, implies that vascular TNF-α is functionally active and contributes to NO inactivation.

Role of tumour necrosis factor-α on endothelin-1/nitric oxide imbalance

In vessels from Obese, IFX elicited an increased relaxation, consistently with an enhanced NO release. BQ-123 also resulted in a higher relaxing response, resistant to L-NAME, confirming that the reduced NO availability unbalances the ET-1/NO system in favour of ET-1 vasoactive effect. The vascular increased expression of ET-1 and ET$_A$ receptor supports the pre-eminent endogenous ET-1-mediated contracting effect. We demonstrated for the first time that in vessels from Obese, IFX blunted the relaxation to the ET$_A$ antagonist and, concomitantly, restored the inhibition by L-NAME on BQ-123. In contrast, in control experiments, L-NAME inhibited the response to BQ-123, regardless of IFX infusion. Overall, these results demonstrate the critical role played by TNF-α in the pathogenesis of the ET-1/NO imbalance. We previously reported that TNF-α reduces agonist-evoked NO availability in obesity.[6] The present findings extend the role of TNF-α in

endothelial dysfunction through a direct effect on tonic NO release and, consequently, its strict cross-talk with the ET-1 vasoactive tone, providing convincing anatomic and morphological support to this hypothesis. Again, our experiments from PVAT$-$ vessels highlight the anticontractile properties of adipose tissue in physiological conditions, and its switch towards an inflammatory pro-contractile phenotype in obesity. Thus, in control arteries, PVAT removal attenuated NO release, resulting in a greater ET$_A$-antagonist-mediated relaxing effect, and a reduction of the inhibitory effect by L-NAME. In contrast, in Obese, PVAT removal attenuated the IFX- and BQ-123-mediated relaxations and restored the inhibition by L-NAME on ET$_A$ receptor antagonist, consistently with the absence of PVAT-derived TNF-α impact.

ET$_B$ receptor is mainly localized within endothelial cells, promoting NO release and ET-1 clearance.[20] However, it cross-talks with ET$_A$ receptor, being this interaction reinforced by a variety of stimuli.[21] Our experiments revealed a greater ET$_B$ expression within arterial wall of obese than controls. ET$_B$ function was not tested in the present work. Nevertheless, we may hypothesize that ET$_B$ receptors mainly amplify the vasoconstrictor activity of ET$_A$ receptors, as a consequence of a lacking NO-mediated vasodilation. This issue awaits future clarification.
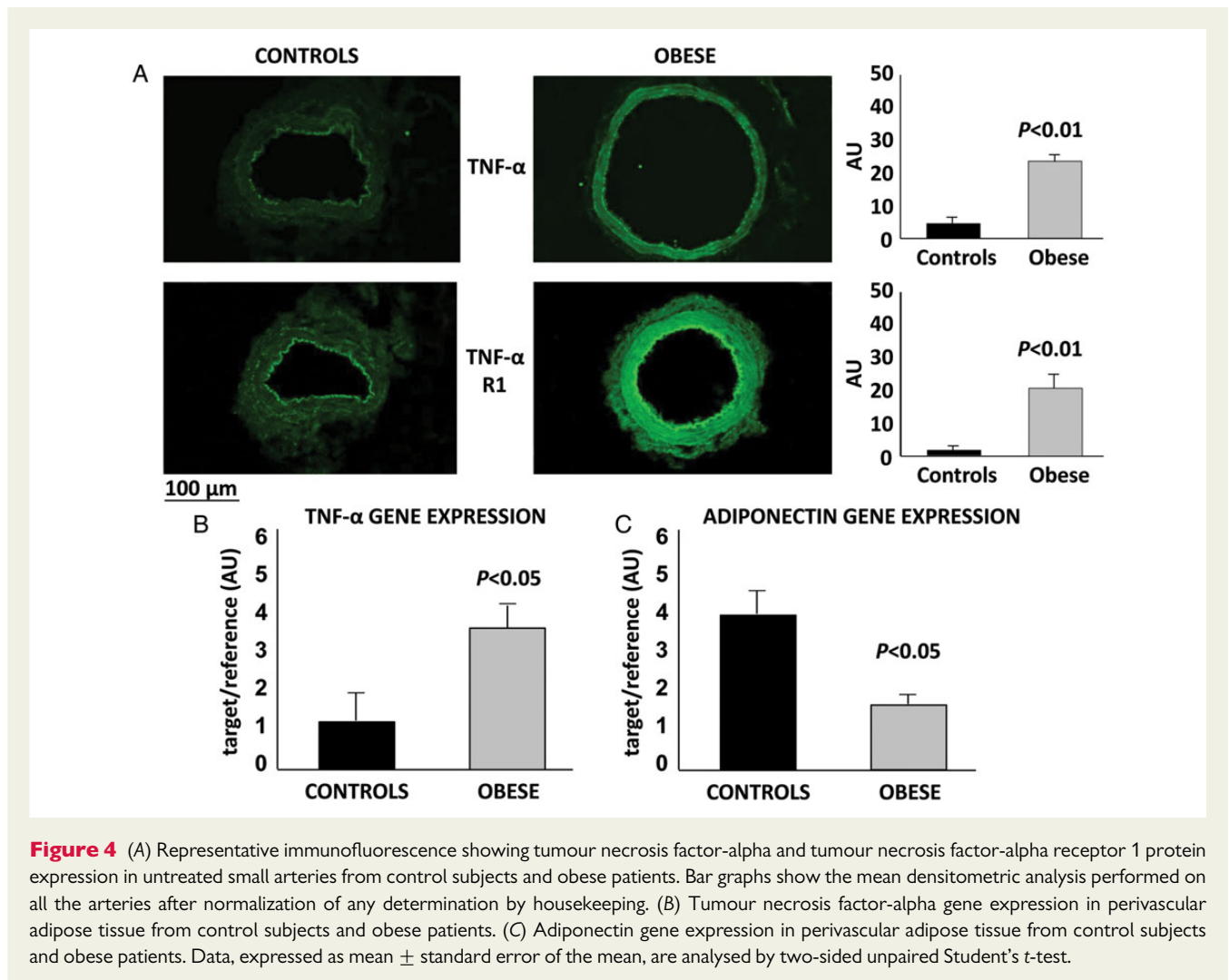
Figure 4 (*A*) Representative immunofluorescence showing tumour necrosis factor-alpha and tumour necrosis factor-alpha receptor 1 protein expression in untreated small arteries from control subjects and obese patients. Bar graphs show the mean densitometric analysis performed on all the arteries after normalization of any determination by housekeeping. (*B*) Tumour necrosis factor-alpha gene expression in perivascular adipose tissue from control subjects and obese patients. (*C*) Adiponectin gene expression in perivascular adipose tissue from control subjects and obese patients. Data, expressed as mean ± standard error of the mean, are analysed by two-sided unpaired Student's *t*-test.

Tumour necrosis factor-α on endothelin-1/nitric oxide imbalance: impact of reactive oxygen species and endothelial nitric oxide synthase uncoupling

The NAD(P)H oxidase enzyme is a major vascular source of ROS in small vessels from obese patients.[6] Such enzymatic complex is also involved in transforming the eNOS from a protective enzyme to a contributor to oxidative stress, the so-called eNOS uncoupling.[22,23] We found that among the Obese group, the increased intravascular superoxide was blunted by L-NAME, thus indicating that the eNOS uncoupling occurs and is involved in ROS generation, concomitantly to reducing NO release. The finding that apocynin without or with L-NAME similarly reduced superoxide generation represents the first demonstration in human obesity that NAD(P)H oxidase acts as a trigger of eNOS uncoupling phenomenon, according to previous animal evidence.[22] In such context, the eNOS uncoupling is a major mechanism whereby TNF-α exerts a deleterious role on the ET-1/NO system. The existence of eNOS uncoupling is strengthened by the detection of a reduced dimeric protein expression, but not the monomeric isoform, among Obese. This hypothesis needs future confirmation even

through other markers for eNOS uncoupling, such as the eNOS S-glutathionylation.[24]

Of noting, the decreased superoxide signal by L-NAME extended throughout the whole vessel wall, suggests that, in addition to eNOS, L-NAME might interfere with other intravascular ROS sources, including inducible (i)NOS, likely upregulated by the inflammatory condition.[6] The abrogated superoxide generation by IFX suggests that in addition to NAD(P)H oxidase, TNF-α promotes ROS generation also through other mechanisms. In such scenario, ET-1 also contributes to generate superoxide, through its ET_A receptor (*Figure 7*).

In our experimental conditions, IFX represents a pharmacological tool highlighting the biological effects of perivascular TNF-α on the whole vascular wall, thus interrupting its activation of the NAD(P)H oxidase system and the ET-1 signalling cascade.

Previous *in vitro* evidence documented that acute TNF-α incubation may stimulate ET-1 generation.[25] Our results showed an increased vascular expression of ET-1 and its receptors, mainly ET_A that accounts for the majority of ET-1-mediated vasoconstrictor effects,[26] in obese group. These data, supported by functional results of an enhanced vasoconstriction to exogenous ET-1, suggest that a direct stimulus for ET-1 generation by TNF-α also occurs. This aspect awaits clarification.
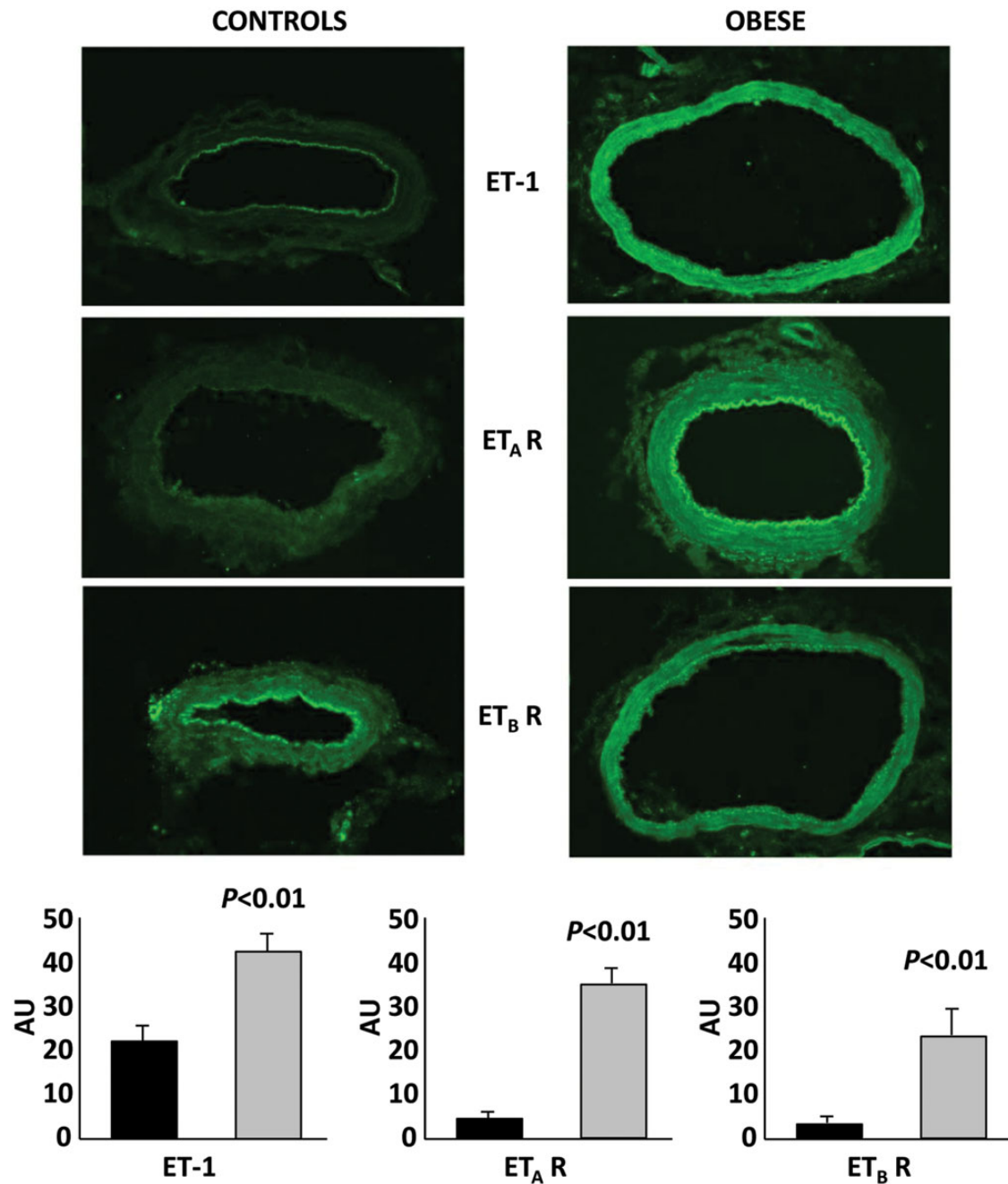
Figure 5 Representative immunofluorescence showing endothelin-1, endothelin-A, and endothelin-B protein expression in untreated small arteries of control subjects and obese patients. Bar graphs show the mean densitometric analysis performed on all the arteries after normalization of any determination by housekeeping in control subjects (black bars) and obese patients (grey bars). Data, expressed as mean $\pm$ standard error of the mean, are analysed by two-sided unpaired Student's t-test.

Intracellular signalling involved in tumour necrosis factor-α-mediated endothelin-1/nitric oxide imbalance

In endothelial cells, TNF-α activates JNK, p38 MAPK, and ERK. While the former two principally regulate the activation of both AP-1 and NF-kB required for TNF-α-induced ET-1 gene expression, ERK has double, somehow contrasting effects, promoting apoptosis and DNA methylation, and enzymatic synthesis.[27] The increased JNK phosphorylation found in Obese agrees with its main role in mediating the functional relationship between TNF-α and ET-1.[28] On the other hand, the reduced p38 MAPK pathway, involved in induction
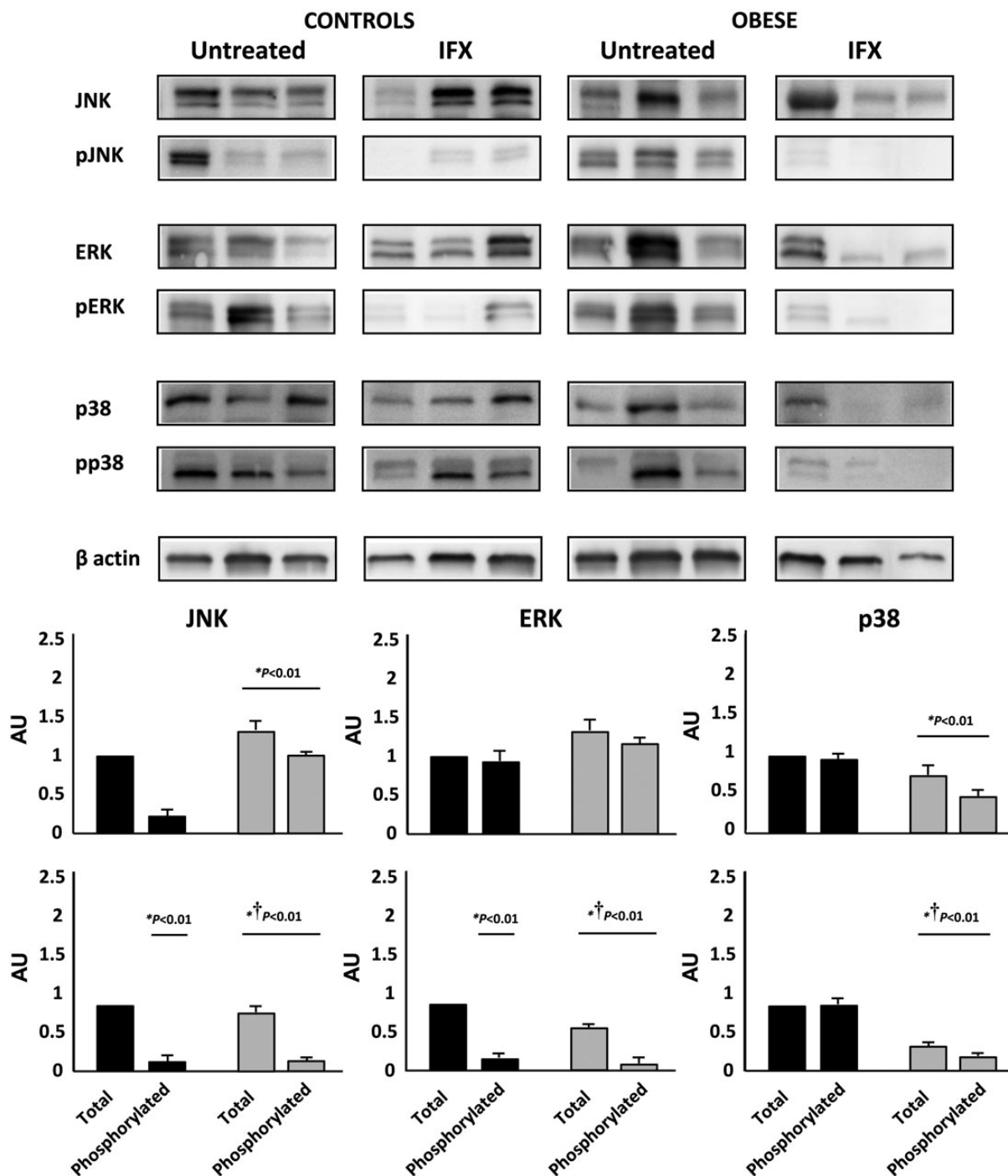
Figure 6 Representative western blot documenting c-Jun N-terminal kinase, extracellular signal-regulated kinase, and p38 expression and phosphorylation in protein extracts from arterial wall of three obese patients (grey bars) and three controls (black bars), before and after incubation with infliximab. Bar graphs show the mean densitometric analysis performed on all the arteries before (upper graphs) and after (lower graphs) treatment with infliximab. Housekeeping-normalized total expression of any kinase in untreated controls is used as internal reference (1 AU). * vs. controls; † vs. untreated.

of key enzymes (cyclooxygenase-2, iNOS, and matrix metalloproteinases), supports the hypothesis of an ET-1-mediated vascular remodelling and a reduced NO production as minor vascular effects of TNF-α.[29] The reduced activation of these kinases by IFX specifically

in Obese points out that TNF-α not only exerts its deleterious effect at the level of endothelium[30] but also affects the direct vasoconstriction exerted by ET-1, mainly via the JNK/pJNK pathway in these patients.
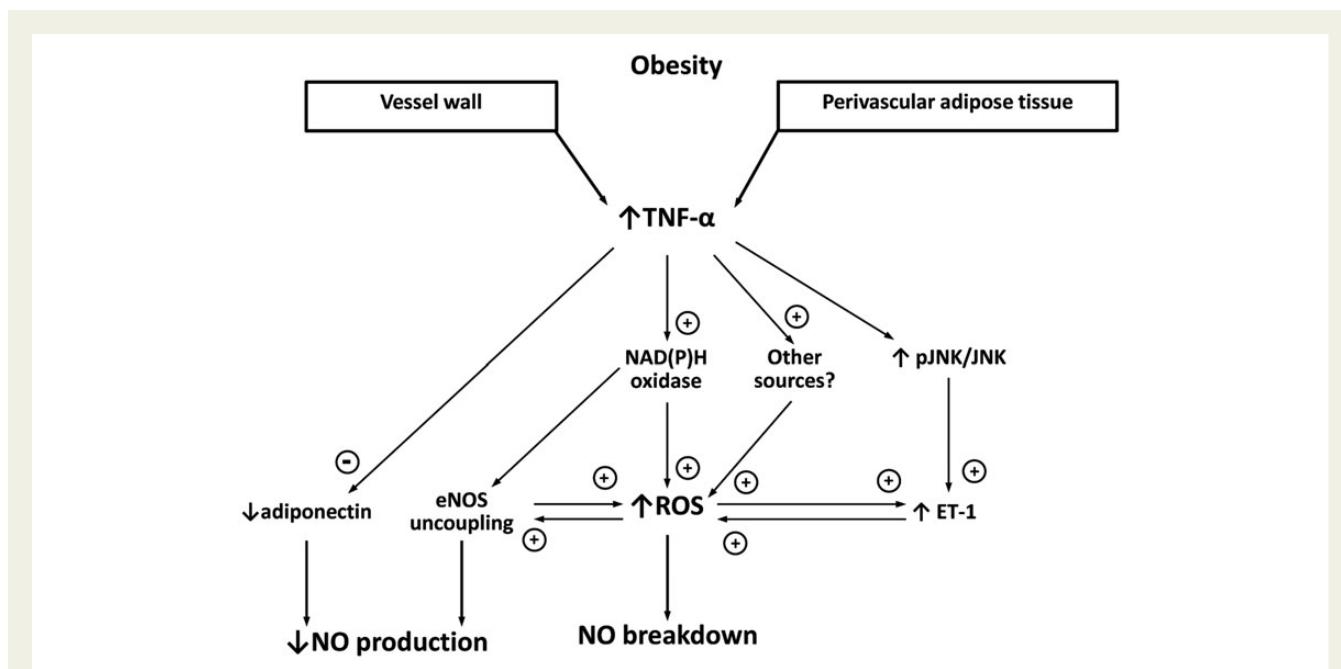
Figure 7 Proposed mechanism whereby tumour necrosis factor-alpha induces endothelin-1/nitric oxide imbalance in small resistance arteries from obese patients. Tumour necrosis factor-alpha-driven vascular NAD(P)H oxidase is a major source of reactive oxygen species, leading to nitric oxide breakdown. Moreover, NAD(P)H oxidase acts as a trigger of endothelial nitric oxide synthase uncoupling, thus potentiating reactive oxygen species generation. Tumour necrosis factor-alpha also directly stimulates endothelin-1 generation via the c-Jun N-terminal kinase pathway, which in turn contributes to reactive oxygen species generation. Reduced adiponectin expression in perivascular adipose tissue may participate in the tumour necrosis factor-alpha-mediated nitric oxide inhibition. Tumour necrosis factor-alpha may promote reactive oxygen species generation through still unknown mechanisms.

Conclusions

In small arteries of Obese, vascular and perivascular TNF-α excess, coupled with an increased vascular expression of ET-1 and ET$_A$ receptor, contribute to the ET-1/NO imbalance. It is proposed that ROS excess, via NAD(P)H oxidase activation, induces the eNOS uncoupling, which in turn contributes to generate superoxide and to impair tonic NO release. The up-regulated JNK pathway may represent a crucial molecular signalling involved in this process. Our results also strengthen the pivotal role of PVAT that, in obesity, loses its vascular protective properties switching towards a functionally active pro-contractile inflammation source.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

Funding

Conflict of interest: none declared.

References

1. Dhaun N, Goddard J, Kohan DE, Pollock DM, Schiffrin EL, Webb DJ. Role of endothelin-1 in clinical hypertension: 20 years on. *Hypertension* 2008;**52**:452–459.
2. Cardillo C, Kilcoyne CM, Cannon RO, Panza JA. Interactions between nitric oxide and endothelin in the regulation of vascular tone of human resistance vessels in vivo. *Hypertension* 2000;**35**:1237–1241.
3. Steinberg HO, Chaker H, Leaming R, Johnson A, Brechtel G, Baron AD. Obesity/insulin resistance is associated with endothelial dysfunction. Implications for the syndrome of insulin resistance. *J Clin Invest* 1996;**97**:2601–2610.
4. Al Suwaidi J, Higano ST, Holmes DR Jr, Lennon R, Lerman A. Obesity is independently associated with coronary endothelial dysfunction in patients with normal or mildly diseased coronary arteries. *J Am Coll Cardiol* 2001;**37**:1523–1528.
5. Grassi G, Seravalle G, Scopelliti F, Dell'Oro R, Fattori L, Quarti-Trevano F, Brambilla G, Schiffrin EL, Mancia G. Structural and functional alterations of subcutaneous small resistance arteries in severe human obesity. *Obesity (Silver Spring)* 2010;**18**:92–98.
6. Virdis A, Santini F, Colucci R, Duranti E, Salvetti G, Rugani I, Segnani C, Anselmino M, Bernardini N, Blandizzi C, Salvetti A, Pinchera A, Taddei S. Vascular generation of tumor necrosis factor-alpha reduces nitric oxide availability in small arteries from visceral fat of obese patients. *J Am Coll Cardiol* 2011;**58**:238–247.
7. De Ciuceis C, Porteri E, Rizzoni D, Corbellini C, La Boria E, Boari GE, Pilu A, Mittempergher F, Di Betta E, Casella C, Nascimbeni R, Rosei CA, Ruggeri G, Caimi L, Rosei EA. Effects of weight loss on structural and functional alterations of subcutaneous small arteries in obese patients. *Hypertension* 2011;**58**:29–36.
8. Cardillo C, Campia U, Iantorno M, Panza JA. Enhanced vascular activity of endogenous endothelin-1 in obese hypertensive patients. *Hypertension* 2004;**43**:36–40.
9. Tesauro M, Schinzari F, Rovella V, Di Daniele N, Lauro D, Mores N, Veneziani A, Cardillo C. Ghrelin restores the endothelin 1/nitric oxide balance in patients with obesity-related metabolic syndrome. *Hypertension* 2009;**54**:995–1000.
10. Dandona P, Aljada A, Chaudhuri A, Mohanty P, Garg R. Metabolic syndrome: a comprehensive perspective based on interactions between obesity, diabetes, and inflammation. *Circulation* 2005;**111**:1448–1454.
11. Virdis A, Neves MF, Duranti E, Bernini G, Taddei S. Microvascular endothelial dysfunction in obesity and hypertension. *Curr Pharm Des* 2013;**19**:2382–2389.
12. Greenstein AS, Khavandi K, Withers SB, Sonoyama K, Clancy O, Jeziorska M, Laing I, Yates AP, Pemberton PW, Malik RA, Heagerty AM. Local inflammation and hypoxia abolish the protective anticontractile properties of perivascular fat in obese patients. *Circulation* 2009;**119**:1661–1670.
13. Dandona P, Ghanim H, Chaudhuri A. An inflammatory tale from 3 fatty depots. *J Am Coll Cardiol* 2011;**58**:256–257.

14. Kahler J, Ewert A, Weckmuller J, Stobbe S, Mittmann C, Koster R, Paul M, Meinertz T, Munzel T. Oxidative stress increases endothelin-1 synthesis in human coronary artery smooth muscle cells. *J Cardiovasc Pharmacol* 2001;**38**:49–57.

15. Pollock DM, Pollock JS. Endothelin and oxidative stress in the vascular system. *Curr Vasc Pharmacol* 2005;**3**:365–367.

16. Endemann DH, Pu Q, De Ciuceis C, Savoia C, Virdis A, Neves MF, Touyz RM, Schiffrin EL. Persistent remodeling of resistance arteries in type 2 diabetic patients on antihypertensive treatment. *Hypertension* 2004;**43**:399–404.

17. Virdis A, Bacca A, Colucci R, Duranti E, Fornai M, Materazzi G, Ippolito C, Bernardini N, Blandizzi C, Bernini G, Taddei S. Endothelial dysfunction in small arteries of essential hypertensive patients: role of cyclooxygenase-2 in oxidative stress generation. *Hypertension* 2013;**62**:337–344.

18. Jimenez-Altayo F, Briones AM, Giraldo J, Planas AM, Salaices M, Vila E. Increased superoxide anion production by interleukin-1beta impairs nitric oxide-mediated relaxation in resistance arteries. *J Pharmacol Exp Ther* 2006;**316**:42–52.

19. Rodbell M. Localization of lipoprotein lipase in fat cells of rat adipose tissue. *J Biol Chem* 1964;**239**:753–755.

20. Johnstrom P, Fryer TD, Richards HK, Harris NG, Barret O, Clark JC, Pickard JD, Davenport AP. Positron emission tomography using 18F-labelled endothelin-1 reveals prevention of binding to cardiac receptors owing to tissue-specific clearance by ET B receptors in vivo. *Br J Pharmacol* 2005;**144**:115–122.

21. Mazzuca MQ, Khalil RA. Vascular endothelin receptor type B: structure, function and dysregulation in vascular disease. *Biochem Pharmacol* 2012;**84**:147–162.

22. Forstermann U, Munzel T. Endothelial nitric oxide synthase in vascular disease: from marvel to menace. *Circulation* 2006;**113**:1708–1714.

23. Munzel T, Gori T, Bruno RM, Taddei S. Is oxidative stress a therapeutic target in cardiovascular disease? *Eur Heart J* 2010;**31**:2741–2748.

24. Chen CA, Wang TY, Varadharaj S, Reyes LA, Hemann C, Talukder MA, Chen YR, Druhan LJ, Zweier JL. S-glutathionylation uncouples eNOS and regulates its cellular and vascular function. *Nature* 2010;**468**:1115–1118.

25. Yang WS, Chang JW, Han NJ, Park SK. Darbepoetin alfa suppresses tumor necrosis factor-alpha-induced endothelin-1 production through antioxidant action in human aortic endothelial cells: role of sialic acid residues. *Free Radic Biol Med* 2011;**50**:1242–1251.

26. Levin ER. Endothelins. *N Engl J Med* 1995;**333**:356–363.

27. Eringa EC, Bakker W, van Hinsbergh VW. Paracrine regulation of vascular tone, inflammation and insulin sensitivity by perivascular adipose tissue. *Vascul Pharmacol* 2012;**56**:204–209.

28. Eringa EC, Stehouwer CD, Walburg K, Clark AD, van Nieuw Amerongen GP, Westerhof N, Sipkema P. Physiological concentrations of insulin induce endothelin-dependent vasoconstriction of skeletal muscle resistance arteries in the presence of tumor necrosis factor-alpha dependence on c-Jun N-terminal kinase. *Arterioscler Thromb Vasc Biol* 2006;**26**:274–280.

29. Merial-Kieny C, Lonchampt M, Coge F, Verwaerde P, Galizzi JP, Boutin JA, Lafontan M, Levens N, Galitzky J, Feletou M. Endothelin-1 inhibits TNF alpha-induced iNOS expression in 3T3-F442A adipocytes. *Br J Pharmacol* 2003;**139**:935–944.

30. Bergh N, Ulfhammer E, Glise K, Jern S, Karlsson L. Influence of TNF-alpha and biomechanical stress on endothelial anti- and prothrombotic genes. *Biochem Biophys Res Commun* 2009;**385**:314–318.

Corrigendum

Corrigendum to: Cardiovascular disease in Europe 2014: epidemiological update. M Nicholas, N Townsend, P Scarborough, and M Rayner. [Eur Heart J. 2014;35:2950-9. doi:10.1093/eurheartj/ehu299]

This article mentioned that 15 616.1 million deaths were caused by CVD in 2010. The correct number is 15 616.1 thousand deaths.