ORIGINAL RESEARCH

Brain-Derived Neurotrophic Factor Expression and Signaling in Different Perivascular Adipose Tissue Depots of Patients With Coronary Artery Disease

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BACKGROUND: Brain-derived neurotrophic factor (BDNF) is expressed in neuronal and nonneuronal cells and may affect vascular functions via its receptor, tropomyosin-related kinase B (TrkB). In this study, we determined the expression of BDNF in different perivascular adipose tissue (PVAT) depots of patients with established coronary atherosclerosis.

METHODS AND RESULTS: Serum, vascular tissue, and PVAT surrounding the proximal aorta (C-PVAT) or internal mammary artery (IMA-PVAT) was obtained from 24 patients (79% men; mean age, 71.7±9.7 years; median body mass index, 27.4±4.8 kg/m²) with coronary atherosclerosis undergoing elective coronary artery bypass surgery. BDNF protein levels were significantly higher in C-PVAT compared with IMA-PVAT, independent of obesity, metabolic syndrome, or systemic biomarkers of inflammation. mRNA transcripts of TrkB, the BDNF receptor, were significantly reduced in aorta compared with IMA. Vessel wall TrkB immunosignals colocalized with cells expressing smooth muscle cell markers, and confocal microscopy and flow cytometry confirmed BDNF receptor expression in human aortic smooth muscle cells. Significantly elevated levels of protein tyrosine phosphatase 1B, a negative regulator of TrkB signaling in the brain, were also observed in C-PVAT. In vitro, inhibition of protein tyrosine phosphatase 1B blunted the effects of BDNF on smooth muscle cell proliferation, migration, differentiation, and collagen production, possibly by upregulation of low-affinity p75 neurotrophin receptors. Expression of nerve growth factor or its receptor tropomyosin-related kinase A did not differ between C-PVAT and IMA-PVAT.

CONCLUSIONS: Elevated expression of BDNF in parallel with local upregulation of negative regulators of neurotrophin signaling in perivascular fat and lower TrkB expression suggest that vascular BDNF signaling is reduced or lost in patients with coronary atherosclerosis.

Key Words: atherosclerosis ■ neurotrophins ■ obesity ■ perivascular adipose tissue ■ tropomyosin-related kinase receptors

Body weight and energy homeostasis are controlled by neuronal regulators in response to factors produced, among others, within adipose tissue. A prototype example is the adipokine leptin, discovered 25 years ago as a major central nervous system regulator of appetite and energy homeostasis derived from fat.¹,²

Neurotrophins are a family of growth factors with key roles in the regulation of the survival, differentiation, and synaptic activity of sympathetic neurons.³ Similar to leptin, neurotrophins are involved in the central nervous system control of food intake and energy metabolism. Mutations⁴,⁵ or haploinsufficiency⁶ of brain-derived neurotrophic factor (BDNF) or its receptor tropomyosin-related kinase (Trk) B⁷,⁸ have been found to cause severe obesity in humans and mice, whereas central nervous stimulation with TrkB agonists or BDNF mimetics...
In addition to hypothalamic neurons, BDNF\textsuperscript{11} and nerve growth factor (NGF)\textsuperscript{12} mRNA expression has been detected in white adipocytes and diverse adipose tissue depots, including visceral fat. Elevated serum levels of BDNF\textsuperscript{13} and NGF\textsuperscript{14} have been reported in overweight and obese but otherwise healthy women, and circulating BDNF levels decreased following weight loss.\textsuperscript{13,15} In addition to body mass index (BMI) and fat mass, elevated circulating BDNF levels were found to correlate with biomarkers of increased cardiovascular risk, such as diastolic blood pressure and dyslipidemia.\textsuperscript{16}

Obesity is an established cardiovascular risk factor, and previous studies have examined differences in circulating BDNF levels in patients with cardiovascular risk factors or disease. Serum BDNF concentrations were reduced in patients with coronary heart disease,\textsuperscript{17} and lower plasma BDNF and NGF levels were observed in patients with angina pectoris\textsuperscript{18} and acute coronary syndromes\textsuperscript{19} or found to predict adverse cardiovascular events.\textsuperscript{20,21} On the other hand, BDNF levels were increased in coronary sinus of patients with instable angina compared with those in aorta and peripheral vein, suggesting that circulating BDNF levels may not accurately reflect its cardiovascular activities.\textsuperscript{22} The short half-life of BDNF in the circulation and poor tissue penetration also suggest that it may primarily exert local activities.\textsuperscript{23} In this regard, overexpression of BDNF was observed in human and mouse atherosclerotic plaques.\textsuperscript{22,24}

Neurotrophins and their receptors are expressed on endothelial cells\textsuperscript{25} and vascular smooth muscle cells (SMCs),\textsuperscript{26} and their expression is upregulated following vascular injury.\textsuperscript{27} Stimulation of SMCs in culture with recombinant BDNF was shown to increase proliferation,\textsuperscript{28} migration,\textsuperscript{27} and matrix metalloproteinase activity.\textsuperscript{28} Others found BDNF to significantly enhance nicotinamide adenine dinucleotide phosphate oxidase activity and to increase reactive oxygen species generation in cultured human coronary artery SMCs.\textsuperscript{22}

Obesity is associated with expansion and detrimental alterations of perivascular adipose tissue (PVAT).\textsuperscript{29} Several cytokines and growth factors have been identified as potential mediators of local effects of PVAT on the vessel wall, including leptin\textsuperscript{30,31} and tumor necrosis factor α (TNF-α).\textsuperscript{32} How obesity or atherosclerosis in the neighboring vessel wall affect the local expression of neurotrophins in perivascular fat has not been examined to date. Here, we examined the expression of neurotrophins, their receptors, and factors affecting neurotrophin signal transduction in PVAT surrounding vessels affected by atherosclerosis compared with PVAT surrounding the internal mammary artery (IMA-PVAT) of patients with established coronary artery disease (CAD).
METHODS

Data that support the findings of this study are available from the corresponding author upon reasonable request. Because of the sensitive nature of the data collected for this study, requests from qualified researchers trained in human subject confidentiality protocols may be sent to the corresponding author.

Study Patients

Between September 2015 and July 2017, 24 patients (79% men; mean age, 71.7±9.7 years; mean BMI, 27.4±4.8 kg/m²) undergoing elective coronary artery bypass graft surgery at the University Medical Center Mainz were included in the study. All patients diagnosed with multivessel CAD by coronary angiography were included in the study. Only patients who refused or were unable to sign the written consent were excluded from the study. Patients in whom paired biomaterial samples were not available or incomplete were also excluded. The study complied with the Declaration of Helsinki and was approved by the university’s ethics committee (number 837.048.15 [9816]). All patients gave prior written informed consent.

Type 2 diabetes mellitus was defined as fasting blood glucose >126 mg/dL, glycated hemoglobin >6.5%, or use of antidiabetic medication. Arterial hypertension was defined as systolic blood pressure >140 mm Hg and/or diastolic blood pressure >90 mm Hg or use of antihypertensive medication. Dyslipidemia was defined as low-density lipoprotein cholesterol >115 mg/dL, high-density lipoprotein cholesterol <40 mg/dL, and/or triglyceride level >190 mg/dL or the intake of lipid-lowering medication. Metabolic syndrome (MetS) was defined as diabetes mellitus plus ≥2 metabolic disorders, including BMI >30 kg/m², arterial hypertension, or dyslipidemia, according to the National Heart, Lung, and Blood Institute.33

Tissue Collection and Processing

Tissues were obtained during coronary artery bypass graft surgery. PVAT was obtained from two representative locations: (1) the fat tissue surrounding the IMA (IMA-PVAT) and (2) the fat directly adjacent to the ascending aorta, where the proximal anastomosis of the IMA bypass graft was connected (C-PVAT). Moreover, a part of the IMA graft and the tissue obtained after preparing the aortal punch were collected. All specimens were kept in sterile saline solution on ice, transferred to our laboratory, and immediately processed for total RNA (using TRIlzol Reagent, Life Technologies) and protein isolation (using lysis buffer containing 1% Triton X-100, 150 mmol/L NaCl, 50 mmol/L Tris-HCl, 5 mmol/L ethylenediaminetetraacetic acid, and phosphatase/and protease inhibitors (ThermoFisher Scientific), as previously described in more detail.31 In some patients, C-PVAT and IMA-PVAT was paraffin-embedded and prepared for histological studies. From all study participants, venous blood was drawn on the day before surgery. Serum was prepared by centrifugation for 10 minutes at 845 rcf, and the supernatant carefully removed, aliquoted, and stored at −80°C pending analysis.

Enzyme-Linked Immunoassays

Protein levels of BDNF (ABIN2708077, sensitivity: 2 pg/mL; detection range: 31.2–2000 pg/mL), NGF (ABIN1874220, sensitivity: 0.95 ng/mL; detection range: 3.12–200 pg/mL), leptin (ABIN414890, sensitivity: 0.059 ng/mL; detection range: 0.156–10 ng/mL), TNF-α (ABIN2540082, sensitivity: 0.66 pg/mL; detection range: 1.56–100 pg/mL), and protein tyrosine phosphatase 1B (PTP1B; ABIN1572089, sensitivity: defined by the manufacturer as the lowest protein concentration that could be differentiated from zero; detection range: 0.156–10 ng/mL) in serum, and PVAT tissue lysates were measured using 96-well plate ELISA kits following the manufacturer’s instructions (antikoerper-online.de).

A standard amount of total protein (330 µg in 100 µL) was loaded into each well. Results are expressed as nanograms of BDNF, NGF, leptin, TNF-α, or PTP1B protein per milligram of total protein in that sample or per milliliter of sample volume (for serum only).

Histology and Immunohistochemistry

Paraffin-embedded sections through human PVAT and vascular tissues (IMA, aortic wall punch) were stained according to the Masson trichrome protocol to simultaneously detect SMCs (red) and extracellular matrix (blue). Antibodies directed against BDNF (clone EPR1292, abcam; ab108319) and NGF (abcam, ab52918) were used to visualize neurophin expression. Antibodies against TrkB (Santa Cruz Biotechnology, sc-8316), human smooth muscle α-actin (SMA; clone IA1, Dako; M0851), and CD68 (Dako, M067601-2) were employed on immediately neighboring serial cross-sections to determine expression of the BDNF receptor in SMCs or macrophages, respectively. Stained sections were photographed on an Olympus BX51 microscope equipped with an Olympus DP73 cooled, digital color camera.

Cultivation of Human Aortic SMCs

Human aortic SMCs (HASMCs) were purchased from PromoCell (C-12532) and cultivated in Smooth Muscle Cell Growth Medium 2 (PromoCell, C-22062), containing 5% fetal calf serum as well as insulin (5 µg/mL), epidermal growth factor (0.5 ng/mL), and basic fibroblast growth factor (2 ng/mL), in a humidified 5% CO₂ atmosphere at 37°C. Cells between passage 2 and 4 were
Reverse Transcriptase and Real-Time Polymerase Chain Reaction Analysis

Total RNA quantity and quality was checked by measuring the absorbance at 260 and 280 nm (BioPhotometer and µGüvette G10, Eppendorf). Using M-MLV Reverse Transcriptase (Promega), 1 µg RNA was reverse transcribed into cDNA. To visualize differences in Trk mRNA levels in aorta and IMA, reverse transcription polymerase chain reaction (PCR) was performed using Go Taq Flexi DNA Polymerase (Promega) and the Eppendorf mastercycler PCR device. To each reaction, 200 ng cDNA was added and subjected to a total of 35 cycles with denaturation at 95°C for 60 seconds, annealing at 60°C for 60 seconds and elongation at 72°C for 60 seconds. Trk receptor mRNA expression was examined using primer pairs against human TrkA (forward: 5′-CAC CAG AGG TCT ACG CCA TC-3′; reverse: 5′-GAA TCC CAA TGC CTC CCT CC-3′), TrkB (forward: 5′-ACT GTG AAA GGC AAC CCC AA-3′; reverse: 5′-GCC ACA TCA ACC AAC AAG CA-3′), TrkC (forward: 5′-GAT GGC TCG AGG TTT CAA AA-3′; reverse: 5′-GGT GAG CCG GTT ACT TGA CA-3′), and p75NTR (forward: 5′-ATC CCT GGC CGT TGG ATT AC-3′; reverse: 5′-GAC AGG GAT GAG GTT GTC GC-3′). Results are quantified by determining the integrated density values of bands on 1.8% agarose gels and normalized to mRNA levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH; forward: 5′-GAC AGC TAC GTG GGT GAC GAA-3′; reverse: 5′-TTT TCC ATG TCG TCC CAG TTG-3′), smooth muscle myosin heavy chain (MYH11; forward: 5′-CAG ATG AGA TGG GCA GTT GT-3′; and collagen type I (COL1A1; forward: 5′-CAG CGC TAT CCA TCA CAG C-3′; reverse: 5′-TTT TGT ATT CAA TCA CTG TCT TGC C-3′). All primers were selected and validated using Primer-BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast) and ordered and manufactured by Eurofins Genomics (Ebersberg, Germany). Quantification was performed using the ΔΔCt method with the reference gene hypoxanthine phospho ribosyl transferase 1 (HPRT1) mRNA for normalization (forward: 5′-GGT ATA AAT TCT TTG CTG ACC TGC TG; reverse: 5′-AAT TAC TTT TAT GTC CCC TGT TGA CTG G′). Results are shown as fold-change versus control (dimethyl sulfoxide)-treated cells (set at 1).

Immunofluorescence Staining and Confocal Microscopy

HASMcs were plated on glass coverslips and grown to subconfluence (80%–90%), fixed with aceton (10 minutes at −20°C, for TrkB) or 4% paraformaldehyde (10 minutes at RT, Sigma-Aldrich; for all other antibodies) for 10 minutes, and permeabilized using 0.25% Triton X-100 for 5 minutes. Unspecific binding sites were blocked by incubation in 1% BSA/PBS for 7 minutes. Next, they were incubated with monoclonal antibodies directed against nonmuscle myosin heavy chain-10 (MYH10; Abcam; ab230823), PCNA (Abcam; ab92552), SMA (Sigma, A2547), TrkB (Santa Cruz Biotechnology, sc-377218), or p75NTR (Abcam, ab221212) followed by Alexa Fluor 488 or 555-labeled secondary antibodies (ThermoFisher Scientific). The cytoskeleton was counterstained using rhodamine phalloidin (Life Technologies) and cell nuclei using DAPI (4′,6-diamidino-2-phenylindole; Roth). Coverslips were mounted with fluorescence mounting medium and cells analyzed at a Leica confocal microscope.

Flow Cytometry Analysis

HASMcs were analyzed using monoclonal (Santa Cruz Biotechnology; catalogue number sc-377218, clone F1) or polyclonal (Santa Cruz Biotechnology; catalogue number sc-8316, H-181) antibodies against TrkB or the monoclonal anti–pan-Trk antibody (Abcam; catalogue number ab181560, clone EPR17341) or monoclonal antibodies against SMA (Sigma Aldrich, clone 1A4) followed by fluorescence-conjugated secondary antibodies (Alexa Fluor 488, MoBiTec). Cells were washed and analyzed on a FACSCanto I (BD Biosciences). Results are expressed as percent positive cells per 1×10⁶ total cells.
Cell Proliferation and Migration Assays

SMC proliferation was examined using the CellTiter 96 AQueous One Solution kit (Promega), following the manufacturer’s instructions. After 24 hours of incubation with either dimethyl sulfoxide, PTP1B inhibitor (50 µmol/L), BDNF (10 ng/mL), or PTP1B inhibitor+BDNF, 20 µL of cellTiter 96 AG one solution reagent was added to each well, and the absorbance at 490 nm read 1 hour later. To study proliferation and migration, the scratch-wound assay was employed, as described. Cells were photographed at baseline (start) and after incubation for 24 hours on a phase-contrast microscope (Motic AE31) and the mean area quantified using ImageJ (version 1.46r, National Institutes of Health).

Statistical Analysis

Results are shown as mean±SD if normally distributed or as median (interquartile range [IQR]) if normal distribution was not present. Normal distribution was tested using the D’Agostino-Pearson normality test. Findings in C-PVAT and IMA-PVAT from the same patient were compared using 2-tailed paired t test or Wilcoxon matched-pairs signed rank test if values were not normally distributed. Findings in PVAT or serum from different patients were compared using unpaired Student t test or Mann-Whitney test if values were not normally distributed. If more than 2 groups were compared, 1-way ANOVA followed by Holm-Sidak multiple comparisons test or Kruskall-Wallis test (if not normally distributed) was employed. Univariate linear regression models were performed to investigate associations between serum BDNF or NGF levels and comorbidities. All statistical analyses were performed using GraphPad Prism version 8.3.1 for Windows (GraphPad Software) and SPSS version 20.0 (SPSS Inc.).

RESULTS

Expression of BDNF and NGF in PVAT of Patients Undergoing Elective Coronary Artery Bypass Surgery

BDNF and NGF protein levels were determined in PVAT surrounding the aortic root and coronary arteries (C-PVAT) of 24 patients with established CAD and compared with IMA-PVAT, a vessel relatively protected against atherosclerotic lesion development. This approach was chosen to minimize confounding effects of systemic or exogenous factors. Baseline clinical parameters of the study collective, including cardiovascular risk factors and current medication, are shown in Table 1, and the results of the preoperative clinical chemistry and whole blood cell count analysis are shown in Table 2.

Quantitative protein expression analysis using specific enzyme-linked immunoassays revealed significantly increased BDNF levels in C-PVAT (5.05±1.68 ng/mg total protein) compared with those in IMA-PVAT (4.07±1.14 ng/mg total protein, P<0.0096) (Figure 1A). BDNF protein levels were significantly higher in men (n=20) than in women (n=4), both in C-PVAT (5.18±1.68 versus 4.38±1.78 ng/mg total protein, respectively; P<0.0001) and IMA-PVAT (4.09±0.98 versus 4.01±1.97 ng/mg total protein, respectively; P<0.0001). Protein levels of NGF were lower and did not significantly differ between C-PVAT and IMA-PVAT (2.81±1.82 versus 2.22 ng/mg total protein [interquartile range, 1.40–2.79], P=0.5549 as determined using Wilcoxon matched-pairs signed rank test) (Figure 1B). Analysis of BDNF mRNA levels also revealed a nonsignificant trend towards higher values in C-PVAT compared with IMA-PVAT (P=0.0781, n=8 paired samples [data not shown]), in line with increased local gene expression. Immunohistochemical analysis confirmed higher BDNF protein levels in C-PVAT versus IMA-PVAT (Figure 1C), whereas NGF immunosignals were weak and similar in both PVAT depots (Figure 1D). ELISA and quantitative PCR analysis of the “classical” adipokine leptin revealed increased leptin mRNA (P=0.0512 versus IMA-PVAT, n=8 paired samples [not shown]) and protein (P=0.0403 versus IMA-PVAT, n=17 paired samples (Figure 1E) levels in C-PVAT compared with IMA-PVAT, confirming a previous report from our group in a different CAD patient cohort. Significantly increased protein levels in C-PVAT compared with IMA-PVAT were also observed for the proinflammatory cytokine TNF-α (P=0.0012) (Figure 1F) and the negative regulator of tyrosine kinase signaling, PTP1B (P<0.0001) (Figure 1G).

Comparison of Perivascular and Circulating Neurotrophin Levels

Comparison of local neurotrophin expression levels to those circulating in serum revealed that BDNF levels were significantly higher in serum compared with those in C-PVAT and IMA-PVAT (13.4 ng/mg total protein [IQR, 10.66–18.65] and 11.14 ng/mL [IQR, 9.77–15.80], respectively; P<0.0001 for both [not shown]). Leptin protein levels were also higher in serum (13.99 ng/mg [IQR, 8.50–15.90] or 14.62 ng/mL [IQR, 7.55–22.05]) compared with C-PVAT or IMA-PVAT (P<0.0001 for both [not shown]). In contrast to BDNF, NGF levels in serum were lower than those in PVAT (0.15 ng/mg [IQR, 0.09–0.18] or 0.14 ng/mL [IQR, 0.08–0.18], P<0.0001 versus C-PVAT and IMA-PVAT [not shown]). Please note that results were normalized to the total protein content (and not sample volume) to be able to compare levels in PVAT with those in serum. Serum values are also given per mL.
Effects of Body Weight and Metabolic Inflammation on Systemic and PVAT Neurotrophin Levels

Previous studies reported elevated circulating neurotrophin levels in obesity, but whether and how obesity alters their expression in perivascular fat is unknown. As shown in Table 3, no significant correlation was observed between BDNF protein levels and BMI, neither in C-PVAT (n=24 pairs; \( r^2 = 0.02715, P = 0.4417 \)) or IMA-PVAT (n=24 pairs; \( r^2 = 0.3598, P = 0.04579 \)) and serum (n=20 pairs; \( r^2 = 0.05448, P = 0.3220 \)). Similar findings were obtained for the association between NGF levels and BMI (Table 4). BDNF levels also did not differ in patients with a BMI above the median of 26.6 kg/m² (n=13) compared with those with a BMI <26.6 kg/m² (n=11), neither in C-PVAT (5.06±1.60 versus 5.04±1.85 ng/mg protein, \( P = 0.9767 \)) or IMA-PVAT (3.96±1.11 versus 4.21±1.22 ng/mg protein, \( P = 0.5961 \)) nor in serum (12.98±4.23 versus 15.83 ng/mg protein [IQR, 11.39–20.18], \( P = 0.1754 \)) (Figure 2A). NGF protein levels were also similar in C-PVAT (\( P = 0.4103 \)) and IMA-PVAT (\( P = 0.6926 \)), but significantly lower in serum of patients with CAD who had a BMI >26.6 kg/m² (\( P = 0.0286 \), Figure 2B).

Classification of patients according to the presence or absence of MetS revealed similar BDNF and NGF protein levels in patients with CAD and MetS (n=9) compared with levels in patients without MetS (n=15), in IMA-PVAT (\( P = 0.3598 \) and \( P = 0.1573 \), respectively), C-PVAT (\( P = 0.4579 \)) and serum (\( P = 0.9680 \) and \( P = 0.2059 \), respectively) (Figure 2C and 2D). Of note, serum glucose levels did not correlate with serum BDNF (n=20 pairs; \( r^2 = 0.02571, P = 0.4995 \)) or NGF levels (n=19 pairs; \( r^2 = 0.04697, P = 0.3728 \)) (Table 4). Univariate linear regression analysis did not reveal significant associations between MeT and BDNF (\( \beta \) estimate, −0.12; 95% CI −0.49 to −0.63 \( P = 0.628 \)) or NGF (\( \beta \) estimate, 0.32; 95% CI 0.32 to 0.15 \( P = 0.181 \)) serum levels.

Obesity and MetS are frequently associated with inflammation. In this regard, BDNF protein levels did not significantly differ between patients with CAD who had CRP (C-reactive protein) levels above the median of 4.7 mg/L (n=14), in IMA-PVAT (\( P = 0.5790 \)), C-PVAT (\( P = 0.6166 \)) and serum (\( P = 0.9406 \)) from those with CRP levels lower than the median of 4.7 mg/L (Figure 2E). Similar findings were obtained for NGF protein levels in IMA-PVAT and C-PVAT (Figure 2F). In contrast, serum NGF levels were significantly higher in patients with CAD who had serum CRP levels above the median of 4.7 mg/L (\( P = 0.0222 \)). A significant correlation between serum NGF and CRP levels was also Table 1. Baseline Characteristics of Study Participants

| Parameter                  | Absolute Number (Percentage of Total) |
|----------------------------|--------------------------------------|
| No. of patients            | 24                                   |
| Age, y                     | 71.7±9.7                             |
| Men                        | 19 (79.2)                            |
| BMI, kg/m²                 | 27.4±4.8                             |
| Obesity                    | 5 (20.8)                             |
| Diabetes mellitus, type 1  | 0 (0)                                |
| Diabetes mellitus, type 2  | 11 (45.8)                            |
| Arterial hypertension      | 22 (91.7)                            |
| Dyslipidemia               | 24 (100)                             |
| Metabolic syndrome         | 9 (37.5)                             |
| Smoking                    | 14 (58.3)                            |
| Aortic valve stenosis      | 4 (13.8)                             |
| Left ventricular ejection fraction: Normal (>55%) | 16 (66.7) |
| Low grade reduced (45%–54%) | 4 (13.8) |
| Medium grade reduced (30%–44%) | 3 (12.5) |
| High grade reduced (<30%)  | 1 (4.2)                              |
| Positive family history    | 10 (41.7)                            |
| Nitrites                   | 0 (0)                                |
| β-Blocker                  | 15 (62.5)                            |
| ACEI                       | 13 (54.2)                            |
| Calcium antagonist         | 8 (33.3)                             |
| Acetylsalicylic acid       | 22 (91.7)                            |
| P2Y12 antagonist           | 12 (50.0)                            |
| Statins                    | 21 (87.5)                            |

ACEI indicates angiotensin-converting enzyme inhibitor; and BMI body mass index.

Table 2. Preoperative WBC Count and Serum CRP Levels

| Parameter                  | Mean±SD |
|----------------------------|---------|
| No. of patients            | 24      |
| Creatinine, mg/dL          | 1.02±0.26 |
| Glucose, mg/dL             | 121±40.9 |
| Troponin, mg/dL            | 1890±5962 (n=13) |
| Leucocytes, x10^3/µL       | 8.0±2.7 |
| Erythrocytes, x10^12/µL    | 4.5±0.1 |
| Hematocrit, %              | 40.7±5.0 |
| Hemoglobin, g/dL           | 13.7±1.8 |
| Thrombocytes, x10^3/µL     | 254±99.9 (n=23) |
| CRP, mg/dL                 | 8.95±12.06 (n=23) |

Data are shown as mean±SD. CRP indicates C-reactive protein; and WBC, whole blood cell.
observed (n=19 pairs; $r^2=0.2084$, $P=0.0494$), whereas serum NGF did not correlate with total white blood cell counts or serum TNF-α levels (Table 4). No correlation was observed between serum BDNF levels and serum CRP or TNF-α levels or total white blood cell count (Table 3).

Figure 1. Expression of neurotrophins and other adipokines in perivascular adipose tissue (PVAT). Protein levels of brain-derived neurotrophic factor (BDNF; A) and nerve growth factor (NGF; B) in paired samples of PVAT surrounding the A. mammae interna (IMA-PVAT) or the aortic root and the coronary arteries (C-PVAT) of patients with coronary artery disease were determined using ELISAs. BDNF (C) and NGF (D) expression in IMA-PVAT and C-PVAT was studied using immunohistochemistry. Scale bars represent 10 µm (upper panel) or 20 µm (lower panel). Protein levels of the adipokines leptin (E), tumor necrosis factor-alpha (TNF-α; F), and protein tyrosine phosphatase 1B (PTP1B; G) were also examined. The results of the statistical analysis of paired (ie, from the same patient) IMA-PVAT and C-PVAT samples and the number of comparisons are indicated within the graphs.
suggest that BNDF is upregulated in C- PVAT independent of signals present in obesity and metabolic inflammation, whereas NGF levels differed only in serum.

Parameters and BDNF Protein Levels in PVAT and Serum

Parameters and NGF Protein Levels in PVAT and Serum

| Parameter            | BDNF                     | NGF                      |
|----------------------|--------------------------|--------------------------|
|                      | IMA-PVAT                 | C-PVAT                   | Serum       |
| Age, y               | $r^2=1.24\times10^{-5}$  | $r^2=0.0847$             | $r^2=0.0024$ |
|                      | $P=0.9870$               | $P=0.1677$               | $P=0.8376$  |
| BMI, kg/m²           | $r^2=0.0272$             | $r^2=3.5\times10^{-5}$  | $r^2=0.0545$ |
|                      | $P=0.4417$               | $P=0.9781$               | $P=0.3220$  |
| WBC count, $10^3/μL$ | ND                       | ND                       | $r^2=0.1251$ |
|                      |                          |                          | $P=0.1260$  |
| Glucose, mg/dL       | ND                       | ND                       | $r^2=0.0257$ |
|                      |                          |                          | $P=0.4995$  |
| Serum CRP, mg/L      | ND                       | ND                       | $r^2=0.0105$ |
|                      |                          |                          | $P=0.6678$  |
| Serum TNF-α, ng/mg   | ND                       | ND                       | $r^2=0.0291$ |
| total protein        |                          |                          | $P=0.4982$  |

BDNF indicates brain-derived neurotrophic factor; BMI, body mass index; C-PVAT, perivascular adipose tissue surrounding the proximal aorta; CRP, C-reactive protein; IMA-PVAT, perivascular adipose tissue surrounding the internal mammary artery; ND, not determined; PVAT, brain-derived neurotrophic factor; TNF-α, tumor necrosis factor α; and WBC, white blood cell.

These findings, which are summarized in Table 5, suggest that BNDF is upregulated in C-PVAT independent of signals present in obesity and metabolic inflammation, whereas NGF levels differed only in serum and in response to inflammatory cues.

Expression of Trk Receptor Isoforms in Human Vessels With and Without Atherosclerosis

Neurotrophins signal via Trk receptors. Based on the observed significant differences of BDNF levels in PVAT surrounding the heart/ascending aorta and the IMA, expression of the high-affinity BDNF receptor TrkB was examined in the underlying vascular tissues. PCR analysis revealed significantly lower mRNA levels of TrkB in tissue lysates of the aorta obtained from patients with CAD ($n=7$) compared with the IMA ($n=9$, $P=0.0418$) (Figure 3B), whereas mRNA levels of TrkA, the receptor for NGF ($P=0.1538$) (Figure 3A) and mRNA levels of the low-affinity receptor p75NTR ($P=0.2196$) (Figure 3D) did not differ between aorta and IMA. Significantly lower mRNA levels in the aorta compared with the IMA were also observed for TrkC, the receptor for neurotrophin-3 (Figure 3C). To visualize the PCR findings, an agarse gel showing the expression of neurotrophin receptors in 2 representative pairs of aorta and IMA tissue lysates from patients is shown in Figure 3E. Histochemical analysis of human IMA cross-sections and aortic punch samples (results after Masson trichrome stain are shown in Figure 3F, top row) using antibodies against TrkB (Figure 3F, middle row) suggested a strong TrkB expression (brown signal) in the media of the IMA, whereas TrkB immunosignals were less abundant in the aorta. Findings after omission of the first antibody (negative control) are also shown (Figure 3F, bottom row).

Expression of TrkB Receptors in HASMCs

PCR analysis of cultivated HASMCs detected mRNA expression of TrkA, TrkB, and p75NTR, whereas TrkC mRNA levels were undetectable (Figure 4A). Trk receptor isoform expression levels were relatively low (TrkA and TrkB) or undetectable (TrkC and p75NTR) in human endothelial cells. Flow cytometry analysis using antibodies directed against TrkB or all Trk isoforms (pan-Trk) suggested that TrkB is the isoform predominantly expressed in HASMCs (Figure 4B), and TrkB expression was validated using immunofluorescence confocal microscopy (Figure 4C). Parallel staining of neighboring serial sections with antibodies against TrkB and SMA also supported that SMCs are the primary vascular cell type expressing TrkB (Figure 4D), whereas CD68 immunosignals were low and did not colocalize with those of TrkB (Figure 4E). The observed reduced expression of TrkB in tissue samples of human aorta may thus reflect the lower number of SMCs in this vessel compared with the IMA.

Effects of BDNF (Over)Stimulation on HASMCs

Previous studies examined specific effects of neurotrophins on vascular cells. Based on our findings of reduced TrkB expression in the aorta and elevated levels of PTP1B, an inhibitor or tyrosine kinase receptor signaling in the surrounding PVAT, we next examined the response of HASMCs in culture to recombinant human BDNF (10 ng/mL), alone or in the presence of a potent, cell-permeable PTP1B inhibitor to mimic unrestricted

**Table 3.** Associations Between Selected Clinical Parameters and BDNF Protein Levels in PVAT and Serum

**Table 4.** Associations Between Selected Clinical Parameters and NGF Protein Levels in PVAT and Serum

BMI indicates body mass index; C-PVAT, perivascular adipose tissue surrounding the proximal aorta; CRP, C-reactive protein; IMA-PVAT, perivascular adipose tissue surrounding the internal mammary artery; ND, not determined; NGF, nerve growth factor; PVAT, brain-derived neurotrophic factor; TNF-α, tumor necrosis factor α; and WBC, white blood cell.

*Significant differences.
signaling of neurotrophins (and other growth factors). Quantitative real-time PCR analysis revealed that stimulation of HASMCs with BDNF (10 ng/mL) significantly increased mRNA expression of CCND1 (Figure 5A), ACTA2 (Figure 5B), MYH11 (Figure 5C), and COL1A1 (Figure 5D), whereas the expression of the apoptosis markers BAX (not shown), BCL2 (not shown), and TP53 (not shown) was not altered. Of note, PTP1B inhibitors alone significantly increased CCND1 mRNA levels, possibly reflecting increased response to natural BDNF protein or recombinant growth factors present in SMC growth medium. Higher BDNF concentrations (20 ng/mL) were less effective and did not significantly alter
Table 5. Effect of Obesity and Metabolic Inflammation on BDNF and NGF Protein Level

|                      | IMA-PVAT | C-PVAT | Serum |
|----------------------|----------|--------|-------|
|                      | BDNF     | NGF    | BDNF  | NGF  | BDNF  | NGF  |
| BMI >26.6 kg/m²      | …        | …      | …     | …    | …     | ↓    |
| MetS present         | …        | …      | …     | …    | …     | …    |
| CRP >4.7 mg/L        | …        | …      | …     | …    | …     | ↑    |

↓ indicates significantly reduced (P<0.05); ↑, significantly increased (P<0.05); ..., no significant alteration; BDNF, brain-derived neurotrophic factor; BMI, body mass index; C-PVAT, perivascular adipose tissue surrounding the proximal aorta; CRP, C-reactive protein; IMA-PVAT, perivascular adipose tissue surrounding the internal mammary artery; MetS, metabolic syndrome; and NGF, nerve growth factor.

CCND1, ACTA2, MYH11, and COL1A1 mRNA levels. Similarly, inhibition of PTP1B completely abolished the low-dose BDNF-induced significant increase in CCND1 (Figure 5A), ACTA2 (Figure 5B), MYH11 (Figure 5C), and COL1A1 (Figure 5D) mRNA expression. TrkB mRNA expression significantly increased in HASMCs treated with BDNF, independent of the presence of the PTP1B inhibitor (Figure 5E). In contrast, mRNA levels of p75NTR, the low affinity BDNF receptor were not altered in response to BDNF, but significantly increased in HASMCs treated with BDNF together with the PTP1B inhibitor (Figure 5F). Immunofluorescence confocal microscopy analysis of primary murine aortic SMCs confirmed the quantitative PCR findings and showed increased p75NTR immunosignals in cells treated with BDNF in the presence of the PTP1B inhibitor (Figure 5G). Immunosignals for SMA (Figure 6A, top row) were strongly increased and those for MYH10 (Figure 6A, bottom row) reduced in cells treated with BDNF or PTP1B alone, whereas SMA and MYH10 expression was reversed to those in untreated cells if cells were stimulated with BDNF in the presence of a PTP1B inhibitor. Moreover, immunocytochemical analyses of PCNA (Figure 6B, top row) or actin fibers (Figure 6B, bottom row) as well as functional analysis of cell proliferation (Figure 6C) or proliferation/migration using the scratch-wound assay (Figure 6D and 6E) revealed an increased response in cells stimulated with BDNF, which was less prominent or lost in those also treated with a PTP1B inhibitor.

These findings show that BDNF signaling promotes HASMC proliferation, differentiation, and collagen production, and indirectly suggest that these activities are blunted in the presence of TrkB receptor overstimulation and desensitization, as it may occur following local overexpression of BDNF and PTP1B observed in PVAT surrounding the aorta of patients with coronary atherosclerosis compared with PVAT surrounding the IMA (Figure 7). Inhibition of PTP1B did not potentiate but rather abolished the cellular effects of BDNF, possibly involving upregulation of p75NTR.

**DISCUSSION**

The main findings of the present study are that: (1) BDNF and NGF protein were readily detectable in PVAT; (2) BDNF protein levels in PVAT were lower compared with those in serum and higher in C-PVAT compared with IMA-PVAT; (3) NGF protein was primarily detected in PVAT, and no differences between both perivascular fat depots were observed; (4) protein levels of BDNF and NGF in PVAT were not affected by the presence of obesity, MetS, or systemic biomarkers of inflammation; (5) expression of the BDNF receptor TrkB could be localized to SMCs and was significantly reduced in the aorta, whereas mRNA levels of the NGF receptor TrkA did not differ; (6) C-PVAT also expressed higher levels of PTP1B, a negative regulator of tyrosine kinase receptor signaling; and (7) pharmacological inhibition of PTP1B in HASMCs abolished their response to BDNF stimulation in terms of proliferation, migration, differentiation, and collagen production, possibly via upregulation of p75NTR. Taken together, the findings of this clinical-experimental study suggest that reduced vascular BDNF signaling, caused by loss of Trk-positive SMCs or Trk receptor downregulation or secondary to elevated levels of antagonistic phosphatases such as PTP1B, may contribute to the pathophysiology of atherosclerosis and atherosclerotic lesion progression.

The expression of neurotrophins in adipose tissue has been reported, and BDNF gene polymorphisms, resulting in lower BDNF levels or impaired BDNF signaling, are associated with severe childhood-onset obesity. In contrast, elevated circulating BDNF levels have been observed in small samples of overweight and obese healthy adults. A recent meta-analysis found that obese patients exhibit serum BDNF levels similar to those of normal-weight controls, and others reported that obesity itself does not influence serum BDNF levels in adults. In the present study in elderly patients with CAD, BDNF levels in serum did not correlate with BMI. Of note, BMI also did not correlate with serum leptin levels, an established marker of adiposity in the general population, although other
Figure 3. Expression of neurotrophin receptors in human arterial vessels.

mRNA levels of tropomyosin-related kinase (Trk) isoform A (TrkA; A), B (TrkB; B), and C (TrkC; C) and of the low affinity receptor p75NTR (D) were determined using reverse transcription polymerase chain reaction in tissue samples of the A. mammaria interna (IMA; n=9) and the aortic wall (n=7). The results of the statistical analysis are indicated within the graphs. A representative agarose gel showing results in paired samples of IMA and aorta from 2 patients are shown (E). Paraffin-embedded cross-sections through the IMA or aorta were stained using Masson trichrome (MTC) stain (upper row; smooth muscle cells in red) or antibodies against TrkB (middle row; positive immunosignals in brown) (F). The results after omission of the first antibody (negative control; bottom row) are also shown. Scale bars indicate 50 μm. GAPDH indicates glyceraldehyde 3-phosphate dehydrogenase.
factors, such as diet, were found to have a greater influence on serum leptin levels than BMI. Also, the small size of the patient collective and low variation of body weight in this selective cohort (median, 26.6 kg/m² [IQR, 24.6–29.4]) may have precluded the detection of such an association.

Obesity is not only associated with an enlargement of visceral adipose tissue, but also with an increase
in PVAT mass. Experimental and clinical evidence suggests that PVAT actively participates in vascular function and remodeling. For example, our group previously reported differences in the amount of leptin expressed in human PVAT surrounding the aortic root and the IMA, and experimental analyses in mice demonstrated that local overexpression of leptin is sufficient to promote intimal hyperplasia. These and other findings (reviewed in Schäfer et al29) suggest that PVAT actively contributes to disease processes in the underlying vascular wall, although effects in the opposite direction may also occur. However, little is known about the effects of obesity on neurotrophin expression in perivascular fat. In the present study, we observed higher BDNF levels in C-PVAT, ie, fat tissue surrounding an atherosclerosis-prone vessel, whereas those of NGF were not altered. Of note, the Cross-sectional nature of our study and the impossibility to analyze earlier time points or undiseased material did not allow us to directly confirm or exclude the possibility that our findings may reflect preexisting differences between both localizations, as described earlier. A previous postmortem analysis of autopsy cases of humans with advanced coronary atherosclerosis found that NGF levels are decreased in atherosclerotic coronary vascular tissue compared with the surrounding subepicardial fat. Whereas BDNF expression was not examined in that study, others found that BDNF mRNA levels did not significantly differ between advanced human atherosclerotic plaques and normal arterial intima. However, those previous studies limited their analyses to the expression of neurotrophins in the vessel wall, whereas PVAT surrounding different types of arterial vessels were not studied.

Regarding the cellular source of locally elevated BDNF levels, PVAT contains numerous other cell types in addition to adipocytes, including immune cells, myofibroblasts, endothelial cells, and nerve fibers. Previous studies reported BDNF and NGF expression in SMCs, endothelial cells, and immune cells, and enhanced BDNF expression was observed in macrophages and SMCs in the atheromatous intima as well as the adventitia of human atherosclerotic lesions. Underlining the importance of SMCs as a primary source of BDNF in the vasculature, Tie2-mediated BDNF gene deletion in hematopoietic cells failed to alter atherosclerotic lesion formation in apolipoprotein E–deficient mice. Megakaryocytes and activated platelets have been identified as a major source of BDNF in human serum. The higher vascularization of C-PVAT, reported previously, and release of BDNF following adherence and activation of platelets to dysfunctional endothelial cells may explain the elevated BDNF levels observed in C-PVAT compared with IMA-PVAT in our study, although this could not be directly examined. Increased levels of inflammation and hypoxia in C-PVAT compared with IMA-PVAT may also have contributed to the observed elevation in local BDNF levels, similar to leptin or lymphangiogenic growth factors. In vitro, proinflammatory cytokines, such as TNF-α, and hypoxia have been shown to upregulate the expression of neurotrophins, and both are present at increased levels in C-PVAT, as shown in the present and a previous study.

The increased expression of BDNF in C-PVAT observed in our study suggests increased local BDNF signaling. Whereas the activity of BDNF on neuronal cells has been extensively studied, much less is known about the action of BDNF on vascular cells. Hypoxia was shown to induce the expression of BDNF and its receptor TrkB, resulting in SMC proliferation, or increased extracellular matrix protein expression and matrix metalloproteinase 2 and matrix metalloproteinase 9 activities in fibroblasts. Others have found stimulation of cultured human coronary artery SMCs with recombinant BDNF to significantly enhance nicotinamide adenine dinucleotide phosphate oxidase activity and reactive oxygen species generation. Findings of increased BDNF release from SMCs in patients with pulmonal-arterial hypertension, a chronic disease characterized by media thickening and perivascular fibrosis, support the pathophysiological relevance of local BDNF overexpression. On the other hand, protective effects of TrkB-mediated BDNF signaling also have been reported. For example, BDNF deficiency was associated with cardiac hemorrhage and subsequent heart failure in mice, and TrkB receptors on perivascular SMCs/pericytes
were shown to promote the survival of myocardial endothelial cells.51 The vascular effects of neurotrophins also depend on their local concentration and whether they are mediated by activation of high-affinity tyrosine kinase receptors or the less selective, low-affinity p75NTR receptor, a member of the tumor necrosis
factor super family. In this regard, previous studies reported dose-dependent effects of BDNF on motor and sensory neurons and decreasing effects with increasing concentrations. The inhibitory actions of high-dose BDNF were found to be mediated by p75NTR, the low-affinity receptor of BDNF mediating opposite cellular effects. Binding of NGF to its receptor TrkA on SMCs was shown to promote matrix metalloproteinase 9 production and migration, whereas NGF binding to p75NTR resulted in SMC apoptosis, myofibroblast proliferation, and fibrosis.

Findings of elevated neurotrophin levels in obesity as well as obesity secondary to reduced neurotrophin expression or activity are reminiscent of leptin and the development of obesity as a consequence of leptin deficiency, mutations of leptin receptors, or other forms of resistance to the weight-reducing effects of the adipokine. Interestingly, Trk-mediated signaling of neurotrophins has been shown to be negatively regulated by PTP1B, a mediator of leptin resistance in obesity. PTP1B-mediated dephosphorylation of the BDNF receptor tyrosine kinase in the central nervous system has been shown to contribute to the development of obesity whereas PTP1B inhibition increased tyrosine phosphorylation of TrkB receptors in mouse brain. The PTP1B-mediated negative regulation of neurotrophin signaling may not only be relevant for the development of obesity but also for phosphoregulation of neurotrophin signaling in the vasculature. However, little is known about the PTP1B-mediated control of Trk receptor signaling during vascular remodeling processes and its importance for the vascular complications of increased body weight. Although we could not mimic the PTP1B overexpression observed in vivo, our findings that pharmacological inhibition of PTP1B in vitro blunts the BDNF-induced effects on SMC proliferation, migration, differentiation, and collagen expression, indirectly underlined the importance of PTP1B in the regulation of BDNF signaling in SMCs. Similar to our findings, other laboratories observed that NGF failed to stimulate dendrite growth following pharmacological inhibition of PTP1B or transfection of neurons with a dominant-negative PTP1B isoform. One possible explanation may be that unrestricted BDNF signaling (in the absence of PTP1B) may result in TrkB receptor downregulation and unresponsiveness to BDNF stimulation. Chronic exposure to BDNF was shown to reduce the responsiveness of rat hippocampal neurons to acute BDNF stimulation by downregulation of TrkB. Although TrkB mRNA levels were similar in HASMCs stimulated with BDNF, alone or in the presence of a PTP1B inhibitor, we observed a strong upregulation of p75NTR mRNA and protein levels only in cells stimulated with BDNF together with a PTP1B inhibitor, a mechanism previously suggested to mediate the inhibitory actions of high-dose BDNF to regulate myofibroblast differentiation and collagen production in response to NGF. Cross-signaling of TrkB with other tyrosine kinase receptors (eg, platelet-derived growth factor receptor) or signaling intermediates (eg, Src kinase) that are known to be modulated by PTP1B and to affect the cellular response may also have played a role. Future studies should dissect in more detail how PTP1B, or its inhibition, may alter BDNF signaling in SMCs.

Our study also has some limitations that need to be mentioned. The small number of patients included in this study cohort limits the types of statistical analyses that can be performed and prevents strong conclusions from being made. The small number of patients included in this study cohort also did not reach the minimum required sample size to perform a multivariate linear regression analysis. In addition, material could only be obtained from patients undergoing surgery and therefore represents a cross-sectional analysis at an advanced stage of the disease. For the same reasons, no time curve analysis could be performed and material from age-matched healthy individuals also could not be obtained and studied in comparison. Although we cannot exclude that medication, external factors, or manipulation during surgery and during material processing may have affected our findings, the paired comparison of tissue from the same patient should minimize the influence of systemic factors. The complex reciprocal cross-talk between PVAT and the vessel wall may also have influenced our findings.

Figure 5. Effects of brain-derived neurotrophic factor (BDNF) stimulation on human aortic smooth muscle cells (HASMCs) and importance of protein tyrosine phosphatase 1B (PTP1B). HASMCs were cultivated until subconfluency, incubated with PTP1B inhibitor (50 µmol/L) or dimethyl sulfoxide (DMSO) for 1 hour before being stimulated with recombinant human BDNF (10 and 20 ng/mL) for an additional 24 hours. RNA was isolated and changes in mRNA expression of cyclin D1 (CCND1; A), smooth muscle α-actin (ACTA2; B), smooth muscle myosin heavy chain (MYH11; C), COL1A1 (D), tropomyosin kinase B (TrkB; E), and p75NTR (F) examined using real-time polymerase chain reaction. *P<0.05, **P<0.01, and ***P<0.001 vs DMSO; #P<0.05 and ##P<0.01 vs PTP1B alone and ¶P<0.05, ¶¶P<0.01, ¶¶¶P<0.001, and ¶¶¶¶P<0.0001 vs BDNF (10 ng/mL), as determined using 1-way ANOVA (A, D, E, and F) or Kruskal-Wallis test (B and C) followed by multiple comparisons tests. Confocal microscopy images after immunostaining of HASMCs with antibodies against p75NTR (green signal). Scale bars indicate 20 μm. DAPI indicates 4',6-diamidino-2-phenylindole.
Figure 6. Morphological and functional response of human aortic smooth muscle cells (HASMCs) to brain-derived neurotrophic factor (BDNF) stimulation and importance of protein tyrosine phosphatase 1B (PTP1B).

HASMCs were cultivated on gelatin-coated coverslips and treated with PTP1B inhibitor (50 µmol/L) or dimethyl sulfoxide (DMSO) for 1 hour before being stimulated with recombinant human BDNF (10 ng/mL) for an additional 24 hours. Cells were fixed and changes in the expression of smooth muscle α-actin (SMA; A, top row) or MYH10 (A, bottom row) or proliferating cell nuclear antigen (PCNA; B, top row; arrows point to positive cells) and actin fiber arrangement (F-actin; B, bottom row) examined using confocal fluorescence microscopy. Scale bars indicate 40 µm (A and B, bottom row) or 20 µm (B, top row). Cell proliferation was analyzed using the CellTiter 96 AQueous One Solution cell proliferation assay (C) and cell proliferation and migration using the scratch-wound assay (D and E). *P<0.05, **P<0.01, and ***P<0.0001 vs dimethyl sulfoxide (DMSO) and ****P<0.01 vs BDNF alone, as determined using 1-way ANOVA followed by multiple comparisons test.
CONCLUSIONS

Taken together, our findings in patients with CAD reveal increased BDNF expression in PVAT surrounding the aorta of patients with coronary atherosclerosis compared with PVAT surrounding the IMA, a vessel less affected by atherosclerosis, and also suggest that local alterations in BDNF signaling in SMCs may contribute to the pathophysiology of atherosclerosis.

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