Possible Role of Brain-Derived Neurotrophic Factor in the Pathogenesis of Coronary Artery Disease

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Background—The neurotrophin (NT) family, including nerve growth factor NT-3 and brain-derived neurotrophic factor (BDNF), has a critical role in the survival, growth, maintenance, and death of central and peripheral neurons. NTs and their receptors are expressed in atherosclerotic lesions; however, their significance in cardiovascular disease remains unclear.

Methods and Results—To clarify the role of NTs in the pathogenesis of coronary artery disease, NT plasma levels in the aorta, coronary sinus, and peripheral veins of patients with unstable angina (n=38), stable effort angina (n=45), and non–coronary artery disease (n=24) were examined. In addition, regional expression of BDNF in coronary arteries was examined in autopsy cases and patients with angina pectoris by directional coronary atherectomy. The difference in BDNF levels, but not NT-3, between the coronary sinus and aorta was significantly greater in the unstable angina group compared with the stable effort angina and non–coronary artery disease groups. Immunohistochemical investigations demonstrated BDNF expression in the atheromatous intima and adventitia in atherosclerotic coronary arteries. BDNF expression was enhanced in macrophages and smooth muscle cells in atherosclerotic coronary arteries. Stimulation with recombinant BDNF significantly enhanced NAD(P)H oxidase activity and the generation of reactive oxygen species in cultured human coronary artery smooth muscle cells.

Conclusions—BDNF has an important role in atherogenesis and plaque instability via the activation of NAD(P)H oxidase. **(*Circulation*. 2005;112:2114-2120.)**

Key Words: circulation ■ coronary disease ■ free radicals ■ nervous system ■ stress

On January 17, 1995, the great Hanshin-Awaji earthquake hit Kobe, Japan, killing 6433 people. Thereafter, there was an increase in mortality from cardiac disease.[1] Chronic psychological stress appears to have an important role in cardiovascular diseases after traumatic events such as a major earthquake.[2] Psychological factors such as depression and acute and chronic stress are potent risk factors for coronary artery disease (CAD).[2] The precise mechanisms by which psychological factors cause cardiovascular disease, however, remain to be determined. Under psychological stress, the hypothalamus-pituitary-adrenal axis and sympathetic nerve system are activated, and a wide range of neurohumoral factors are dynamically regulated, including neurotrophins (NTs).

NTs form a family of dimeric polypeptides, which include nerve growth factor, brain-derived neurotrophic factor (BDNF), NT-3, and NT-4/5 in humans.[3–5] NTs have critical roles in the survival, growth, maintenance, and death of central and peripheral neurons.[5,6] Under psychological stress, the secretion of NTs from the hypothalamus, pituitary gland, and central and peripheral nerves is markedly altered.[7] The biological activities of NTs are mediated via the specific high-affinity receptors trkA, trkB, and trkC and the low-affinity NT receptor p75.[8,9] NTs and their receptors are expressed in nonneuronal tissues and various cell types such as developing heart,[10] spleen,[11] atherosclerotic vessels,[12] macrophages,[13] lymphocytes,[14] endothelial cells,[15] and vascular smooth muscle cells,[12] suggesting that NTs have diverse roles even in nonneural organs. The significance of NTs in cardiovascular disease remains to be elucidated.

Acute coronary syndrome occurs as a consequence of coronary plaque rupture and superimposed thrombus. Reactive oxygen species derived from NAD(P)H oxidase have a critical role in the pathogenesis of CAD and plaque instabil-

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TABLE 1. Patient Characteristics

	Non-CAD (n=24)	SAP (n=45)	UAP (n=38)	*P*
Age, mean (range), y	62 (43–78)	65 (46–85)	66 (53–78)	0.36
Male sex, %	63	67	66	0.94
Degree of coronary stenosis, %		88.6 (74.8–94.3)	91.2 (74.6–95.0)	0.63
Ejection fraction, %	63 (51–77)	55 (40–69)	60 (41–70)	0.52
Hypertension, n (%)	12 (50)	32 (71)	20 (53)	0.13
Diabetes, n (%)	10 (42)	22 (49)	19 (50)	0.80
Hyperlipidemia, n (%)	9 (38)	21 (47)	20 (53)	0.62
Smoking, n (%)	10 (42)	19 (42)	17 (45)	0.96
Obesity, n (%)	9 (38)	20 (44)	15 (40)	0.83

Degree of coronary stenosis and ejection fraction are given as medians and interquartile ranges. Numbers of diseased vessels are given as mean and SEM.

ity.[16] BDNF induces oxidative stress via the activation of this oxidase system in cortical cells.[17] The localization of NTs in the cardiovascular system and their potent biological activities suggest a possible role for these neurotrophic molecules in the pathogenesis of cardiovascular disease, including acute coronary syndrome. To clarify the significance of NTs in the pathogenesis of CAD, we examined NT plasma levels in the coronary circulation of patients with angina pectoris and non-CAD and their regional expression in coronary arteries obtained from autopsied cases and coronary specimens obtained during directional coronary atherectomy (DCA). Furthermore, we examined the pro-oxidative effects of NTs on cultured vascular cells.

Methods

Patient Groups

Patients who underwent diagnostic coronary angiography and patients with angina in whom significant stenosis of the left coronary arteries was documented were enrolled. Subjects were divided into unstable angina (UAP), stable effort angina (SAP), and non-CAD groups. Table 1 shows the clinical characteristics of the 3 groups. The UAP group consisted of 38 patients who had anginal episodes at rest or angina during a mild degree of effort within 48 hours of the study without a significant increase in creatine phosphokinase levels. Patients were classified IB (n=14), IIB (n=12), and IIIB (n=12) according to Braunwald's criteria. The SAP group consisted of 45 patients with typical effort angina or positive treadmill exercise testing but no episodes of angina at rest. All patients with angina had >75% stenotic lesions in the left coronary artery determined by myocardial perfusion scintigraphy to be the culprit lesion. The non-CAD group consisted of 24 patients with chest pain syndrome (n=22) or mitral valve prolapse (n=2). They had no significant coronary artery stenosis >25% luminal diameter. No patients had acute infection, acute inflammation, or psychological disorders. No patients had taken antidepressant drugs, major tranquilizers, steroids, or nonsteroidal antiinflammatory drugs except for aspirin. Written informed consent was obtained from all patients before enrollment in the study.

Human Blood Samples

Before the injection of a contrast medium, blood samples were collected from the coronary sinus (Cs), aortic root (Ao), and femoral vein. At the time of blood sampling, the first 3 mL of blood was discarded, and additional blood was drawn into a tube containing EDTA (pH 7.5) for NT assay. The blood samples were immediately centrifuged at 3000 rpm for 10 minutes at 4°C, and the plasma was stored at −80°C until assayed.

Measurement of Plasma NT Levels

NTs were measured by sandwich ELISAs according to the manufacturer's instructions for BDNF and NT-3 (Promega). Assays were performed on polystyrene 96-well plates. The NT concentration was quantified against a standard curve calibrated with known amounts of protein. The detection limits were 4 pg/mL for BDNF and 8 pg/mL for NT-3. The BDNF or NT-3 ELISA systems have very low cross-reactivity with other related neurotrophic factors: 3% or 0.11% cross-reactivity, respectively. Each value is a mean of duplicated measurement.

Human Tissue and Immunohistochemistry

Human coronary arteries were collected from 11 autopsy cases within 6 hours after death. Table 2 shows the characteristics of the autopsy cases. Coronary arteries were removed from the heart and cut into 3-mm lengths. Before the immunohistochemical analysis, all autopsy sections were examined by hematoxylin and eosin staining and classified into nonatherosclerotic coronary arteries (n=7) and atherosclerotic arteries (n=13). Coronary specimens were obtained from patients with SAP (n=29) or UAP (n=21) during DCA.

Tissue distribution of BDNF was detected through the use of immunohistochemical methods according to the manufacturer's instructions for anti-human BDNF antibody (Chemicon International or Santa Cruz Biotechnology Inc). BDNF antibody has <0.1% cross-reactivity with recombinant human NT-3 or NT-4/5. Human tissues were fixed in Zamboni's fixative (4% formaldehyde, 15% picric acid in 0.1 mol/L phosphate buffer) for 2 hours and in 30% sucrose in PBS overnight at 4°C. Cryostat sections (20 μm) were blocked with 20% normal horse serum in PBS for 1 hour, followed by incubation with primary antibody diluted 1:500 in 2× PBS and

TABLE 2. Characteristics of Autopsy Cases

Sex	Age, y	Cause of Death	Associated Cardiovascular Disease(s)
Male	82	Hepatoma	Stable angina
Male	78	Pancreatic cancer	Hypertension
Male	73	Cardiac rupture	Acute myocardial infarction
Male	70	Congestive heart failure	Old myocardial infarction
Female	66	Hepatoma	Hypertension
Male	65	Lung cancer	None
Female	64	Lymphoma	Hypertension
Male	63	Colon cancer	None
Male	51	Congestive heart failure	Dilated cardiomyopathy
Male	41	Sudden death	Ventricular tachycardia
Male	35	Gastric cancer	None

0.3% Triton X-100 containing 0.02% sodium azide. After a 24-hour incubation at room temperature, the samples were washed and incubated with biotinylated goat anti-rabbit immunoglobulin (DAKO). For color development, we used an LSAB kit (DAKO). For a negative control, the primary antibody was replaced with rabbit serum.

Double-Labeling Immunofluorescence

The antibodies used in double staining were mouse monoclonal anti-human CD68 antibody (DAKO) for macrophages and mouse monoclonal anti-human smooth muscle α-actin antibody (DAKO) for smooth muscle cells. Texas red–conjugated anti-mouse immunoglobulin and FITC-conjugated anti-rabbit immunoglobulin were applied as secondary antibodies. The samples were examined by a laser scanning confocal imaging system (MRC-1024, Bio-Rad Laboratories).

Semiquantitative Analysis of BDNF in Immunohistochemistry

According to a previous study, the expression of BDNF in each DCA specimens was graded as follows: grade 0=negative stain, grade 1=variable or weak stain, and grade 2=moderately or strongly positive stain.[18] The sections were blindly graded by 3 independent senior pathologists.

Measurement of NAD(P)H Oxidase Activity in Human Coronary Artery Smooth Muscle Cells

Human coronary artery smooth muscle cells (CASMCs; Clonetics) were cultured with medium (Clonetics) supplemented with 10% FBS and the manufacturer's reagents (Clonetics).

The enzymatic activity of NAD(P)H oxidase in human CASMC homogenates was assessed by lucigenin-enhanced chemiluminescence (L-CL). Human CASMCs were preincubated with or without 100 ng/mL BDNF for 1 hour; suspended in homogenate buffer containing 50 mmol/L Tris/HCL (pH 7.4), 1.0 mmol/L EDTA, 500 mmol/L phenylmethylsulfonyl fluoride, 2.0 mmol/L leupeptin, and 2.0 mmol/L pepstatin A; and then homogenized with an ultrasonicator (4×15 seconds) on ice. In the time-course study, the cells were incubated for each period with 100 ng/mL BDNF; in the dose-response study, they were stimulated with the indicated concentration of BDNF for 1 hour. The assay solution contained 50 mmol/L HEPES (pH 7.4), 1.0 mmol/L EDTA, 6.5 mmol/L $MgCl_2$, 5.0 μmol/L lucigenin as an electron acceptor, and either 1 mmol/L NADH or 1 mmol/L NADPH as a substrate. After preincubation at 37°C for 10 minutes, the reaction was started by adding 100 μL cell homogenate. The final volume of the reaction solution was 1.0 mL. Photon emission was recorded continuously for 20 minutes. The chemiluminescent signals observed in the absence of homogenates were subtracted from the chemiluminescence signals of the samples. The chemiluminescence signal was corrected for the protein concentration of each cell homogenate and expressed as counts per minute per milligram protein for an average 20-minute period. In some experiments, the cell homogenates were preincubated with 10 μmol/L diphenylene iodonium (DPI), a selective NAD(P)H oxidase inhibitor, for 20 minutes before L-CL measurement.

Superoxide Production From Human CASMCs

Dihydroethidium oxidative fluorescence dye was used to evaluate in situ production of superoxide. Human CASMCs were preincubated with or without 100 ng/mL BDNF for 1 hour, treated with dihydroethidium (10 μmol/L), and then capped with coverslips. The slides were incubated in a light-protected humidified chamber at 37°C for 20 minutes. The dihydroethidium image was obtained by a laser scanning confocal imaging system (MRC-1024) equipped with a 585-nm long-pass filter.

TABLE 3. BDNF and NT-3 Concentration in Cs, Ao, and Peripheral Vein

	Non-CAD, pg/mL	SAP, pg/mL	UAP, pg/mL	*P*
BDNF				
Cs	1308 (822–1711)	1206 (683–1608)	1503 (1123–2350)	0.67
Ao	1347 (781–1743)	1229 (697–1705)	1249 (776–1754)	0.53
V	1297 (654–1856)	1267 (781–1706)	1304 (802–1689)	0.78
NT-3				
Cs	511 (460–555)	459 (380–546)	433 (308–807)	0.51
Ao	498 (345–587)	428 (360–530)	430 (332–753)	0.76
V	492 (387–606)	445 (365–530)	442 (321–799)	0.77

V indicates peripheral vein. All values are given as medians and interquartile ranges. Probability values are for the comparison among the 3 groups.

Statistical Analysis

Data are expressed as medians and interquartile ranges, medians and ranges, or means and SEM as appropriate. Statistical comparison for categorical variables such as risk factors and sex was performed by the χ^2 test. Age and left ventricle ejection fraction were compared between groups with 1-way ANOVA. Statistical comparison for coronary stenosis between the SAP and UAP groups and the NAD(P)H oxidase activity was performed by the nonparametric tests; the Mann-Whitney was used for comparison. Statistical comparison for NT plasma levels was performed by the nonparametric Kruskal-Wallis tests with multiple-comparison post-hoc procedures (Dunn's method). Statistical analysis was performed with StatView 5 software (SAS Instiitute, Inc). A value of $P<0.05$ was considered to indicate statistical significance.

Results

Patient Characteristics

Table 1 shows the clinical characteristics of the 3 groups. There were no statistical differences among the 3 groups in the following variables: age, sex, left ventricular ejection fraction, hypertension, diabetes mellitus, hyperlipidemia, smoking, or obesity. There were no significant differences between the UAP and SAP groups in the number of diseased vessels or the degree of angiographic stenosis. There was no difference in the standard medications between the UAP and SAP patients.

BDNF in Coronary Circulation Was Increased in Unstable Angina

There was no difference in plasma levels of BDNF and NT-3 among the 3 groups (Table 3). To examine coronary circulation–specific levels of NTs, the difference in NT levels between the Cs and Ao was calculated. The Cs-Ao difference in plasma BDNF in the UAP group was significantly greater than that in the SAP and non-CAD groups, whereas there was no Cs-Ao difference in NT-3 among the groups (Figure 1).

TABLE 4. Semiquantitative Analysis of BDNF in Coronary Specimens Obtained by DCA

	Expression Scores
SAP (n=29)	0.57 (0.33–1.33)
UAP (n=21)	1.74 (1.0–2.33)*

For expression scores, negative stain=0, weak stain=1, and moderate or strong stain=2. All values are given as medians and interquartile range.

*Significant differences between UAP and SAP, $P<0.01$.

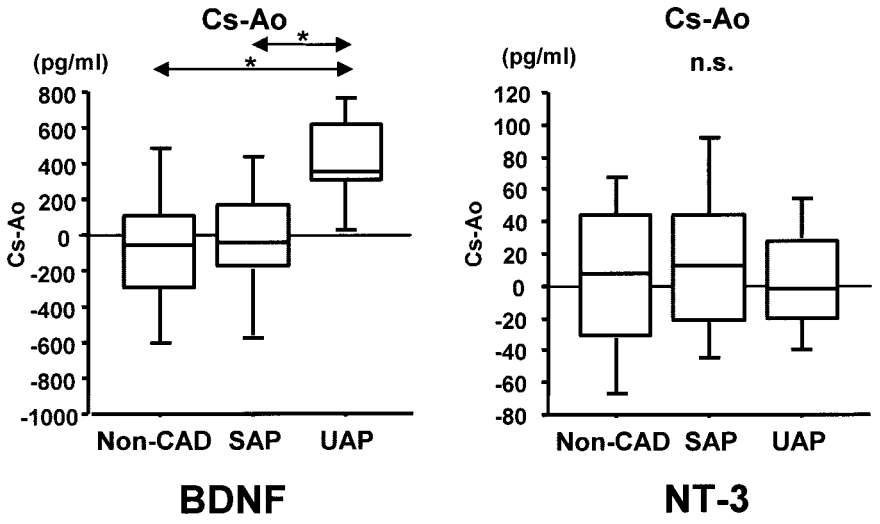

Figure 1. Cs-Ao differences in plasma NT levels across the coronary circulation. The Cs-Ao differences in plasma BDNF were significantly greater in the UAP group than in the SAP or non-CAD group, whereas the Cs-Ao differences in NT-3 were not significantly different among the 3 groups. Data are expressed as medians, with 25th and 75th percentiles (boxes) and 10th and 90th percentiles (I bars). *$P<0.01$ for the comparisons of the UAP, SAP, and non-CAD groups.

These findings indicate that the generation of BDNF in the coronary circulation was enhanced in patients with UAP.

BDNF Expression in Human Coronary Artery

The blood sampling results (Figure 1) led us to examine BDNF expression in the coronary arteries. Immunohistochemical analysis of coronary arteries obtained from autopsy cases was performed. BDNF was expressed in atherosclerotic coronary arteries in all specimens (Figure 2). BDNF was preferentially localized in the atheromatous intima and around the vasa vasorum in the adventitia (Figure 2D and 2E). In contrast, BDNF immunoreactivity was barely detected in nonatherosclerotic coronary arteries (Figure 2C). Figure 2F shows BDNF expression in peripheral nerves. Double staining with cell-specific markers using serial sections revealed that some smooth muscle cells and macrophages expressed BDNF (Figure 3).

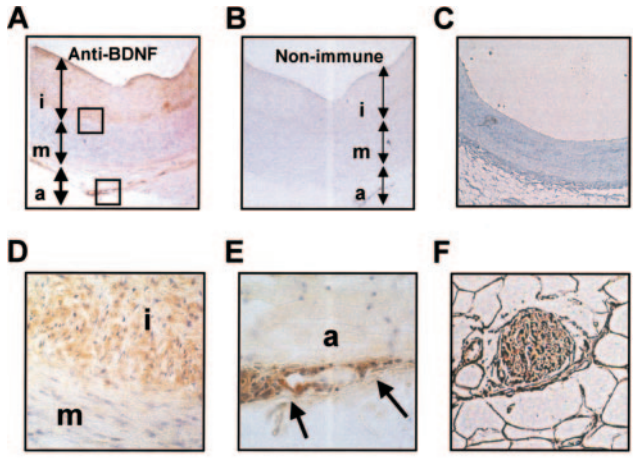

Figure 2. BDNF expression in human coronary arteries of autopsy cases. A, Low-power view of representative human atherosclerotic coronary arteries obtained from autopsied cases shows intense BDNF immunoreactivity in the atherosclerotic intima (i) and adventitia (a). B, There is no significant staining with nonimmune serum used as a control. C, Low-power view of representative nonatherosclerotic coronary arteries shows negligible immunoreactivity of BDNF. D, High-power view of the area indicated by the rectangle in A shows BDNF expression in smooth muscle cells of the intima. E, High-power view of the area indicated by the rectangle in B shows the expression of BDNF in fibroblasts around the vasa vasorum in the adventitia. F, Immunostaining of BDNF in peripheral nerves in pericardial tissues. m Indicates media.

BDNF Expression in Coronary Specimens of Patients With Angina Pectoris

Investigation of coronary specimens from DCA of patients with SAP (n=29) and UAP (n=21) revealed enhanced BDNF expression in inflammatory cells, smooth muscle cells, and extracellular matrix (Figure 4). To investigate the

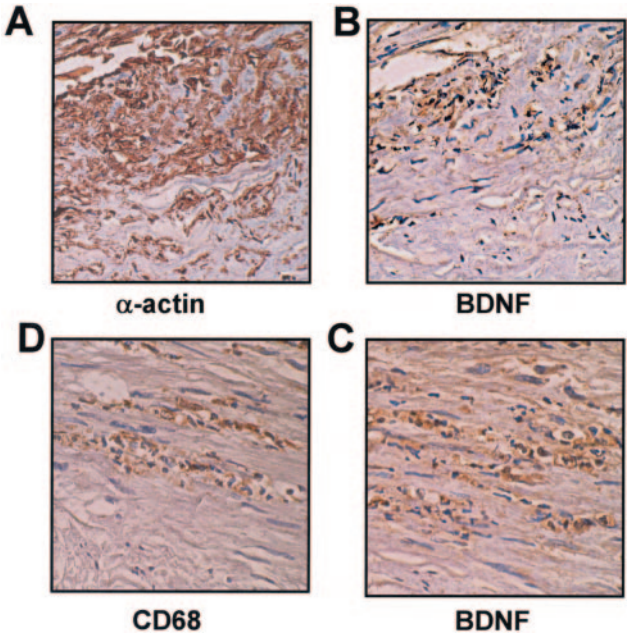

Figure 3. Association between BDNF and CD-68 or α-actin in atherosclerotic coronary arteries. A, B, Immunohistochemical staining of α-actin, a marker of smooth muscle cells (A), and BDNF (B) in serial sections of atherosclerotic coronary arteries of autopsy sample, showing that some smooth muscle cells expressed BDNF. C, D, Immunohistochemical staining of CD-68, a marker of macrophages (C), and BDNF (D) in serial sections of atherosclerotic coronary arteries of autopsy samples, showing that macrophages expressed BDNF.

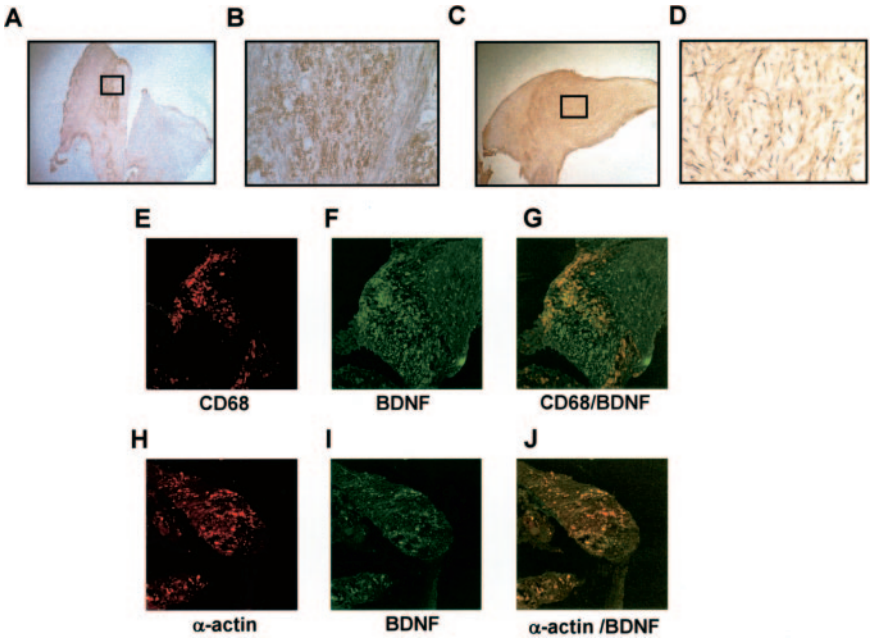

Figure 4. BDNF expression in DCA specimens. Low-power (A, C) and high-power (B, D) views of representative staining for BDNF of DCA specimens from angina patients. Immunohistochemical analysis revealed BDNF expression in inflammatory cells, smooth muscle cells, and extracellular matrix. E–J, Double immunofluorescence of BDNF with cell-specific markers. Anti-CD68 (E) and anti–α-actin (H) were used as markers of macrophage and smooth muscle cells, respectively. Red-labeled immunofluorescence indicates cell makers; green-labeled immunofluorescence indicates expression of BDNF (F, I). Colocalization of cell-specific makers and BDNF is shown by yellow-labeled immunofluorescence (G, J). Representative figure (n=5) is shown, and similar results were observed in all examinations.

cell types of BDNF-expressing cells, double immunofluorescence of BDNF and CD68 or α-actin, a specific marker of macrophages and smooth muscle cells, respectively, was carried out. As shown in Figure 4E through 4J, smooth muscle cells and macrophages expressed BDNF in coronary specimens of patients with angina pectoris.

Semiquantitative analysis demonstrated that BDNF expression in coronary arteries of UAP patients was more intense compared with SAP (Table 4). Representative cases of UAP and SAP are shown in Figure 5.

BDNF Enhanced NAD(P)H Oxidase Activity and Reactive Oxygen Species in Human CASMCs

The effects of BDNF on NAD(P)H oxidase activity were examined in human CASMCs. Stimulation with 100 ng/mL BDNF increased NADH- and NADPH-dependent oxidase activity ≈2.7- and 2.3-fold compared with that in control (nontreated) cells, respectively (Figure 6A). This oxidative activity was reduced by 10 μmol/L DPI, a selective inhibitor of NAD(P)H oxidase. In the presence of DPI, NADH- and NADPH-dependent oxidase activity in CASMCs was reduced by 74% and 45%, respectively. The effects of BDNF on NAD(P)H oxidase activity were dose and time dependent (Figure 6B). Furthermore, in experiments with dihydroethidium, an intracellular fluorescence probe, BDNF stimulation increased the generation of reactive oxygen species (Figure 6C).

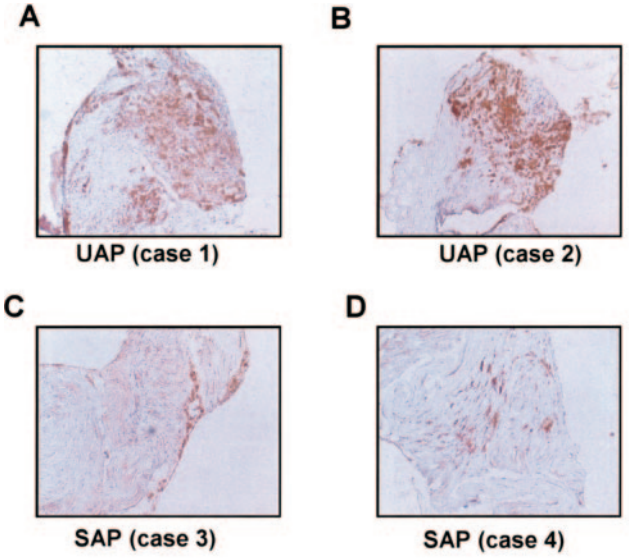

Figure 5. BDNF expression in coronary specimens of patients with angina pectoris. A B, Representative cases of UAP; C, D, representative cases of SAP. Semiquantitative analysis is shown in Table 4.

Discussion

The present study demonstrated a significantly greater Cs-Ao difference in plasma BDNF, but not NT-3, in the UAP group than in the SAP and non-CAD groups. Immunohistochemical analysis revealed that BDNF was expressed in atheromatous intima and adventitia in human coronary arteries. Intense BDNF immunoreactivity was observed in macrophages and smooth muscle cells in atherosclerotic coronary arteries. Semiquantitative analysis demonstrated that BDNF expression in UAP patients was more intense compared with SAP. Furthermore, BDNF enhanced NAD(P)H oxidase activity and superoxide production in cultured CASMCs, and its selective inhibitor suppressed the effect of BDNF. Thus, BDNF in the coronary vasculature might enhance oxidative stress via the activation of NAD(P)H oxidase.

BDNF has protective effects against injury or ischemia in both the central and peripheral nervous systems.[4–6] For example, Schabitz et al[19] demonstrated that intravenous BDNF injection reduces infarct size in rat model. On the other hand, several lines of evidence suggest that NTs potentiate neuronal death under some conditions such as serum or oxygen-glucose deprivation.[20,21] Kim et al[17] demonstrated that BDNF acts as a pronecrotic factor through activation of NAD(P)H oxidase in cortical cells, and other

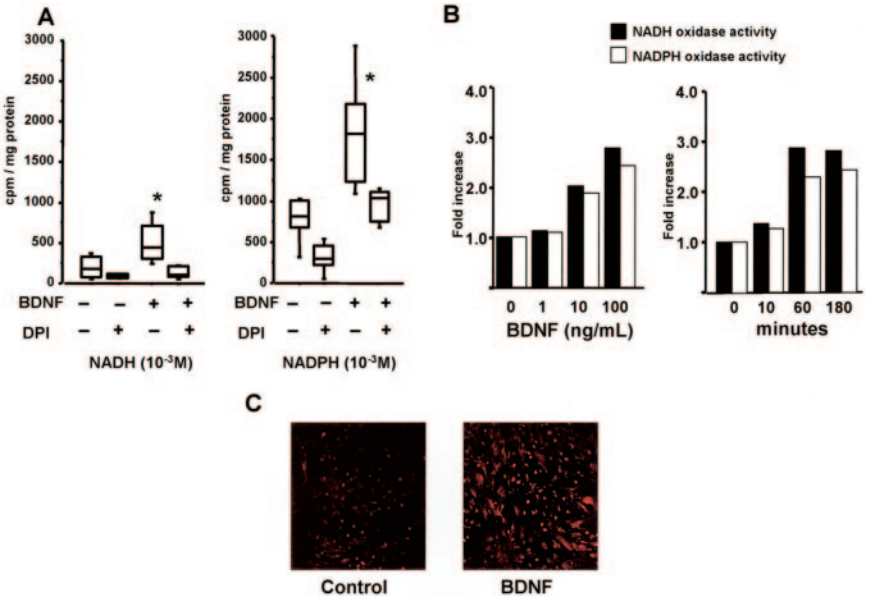

Figure 6. A, Effect of BDNF on NAD(P)H oxidase in cultured human CASMCs. Enzymatic activity of NAD(P)H oxidase in human CASMC homogenates was assessed by L-CL. NADH- (left) and NADPH- (right) dependent oxidase activity was enhanced by treatment with BDNF (100 ng/mL) for 1 hour. Treatment with DPI reduced the activity of NAD(P)H oxidase. The chemiluminescent signals are expressed as counts per minute per milligrams of protein. Data are expressed as medians, with 25th and 75th percentiles (boxes) and 10th and 90th percentiles (I bars). B, Dose response and time dependency of BDNF on NAD(P)H oxidase activity. In the dose-response study, CASMCs were stimulated with the indicated concentration of BDNF for 1 hour (left). In the time-course study, the cells were incubated for each period with 100 ng/mL BDNF (right). Graphs are representatives of 4 independent experiments. *$P<0.05$ for the chemiluminescent signals of CASMCs with and without BDNF preincubation. C, Effect of BDNF on reactive oxygen species generation in cultured human CASMCs. Generation of reactive oxygen species was assessed with the dihydroethidium method. Treatment with BDNF (100 ng/mL) increased superoxide generation vs control. Results are representative of 5 independent experiments.

studies indicated that NTs induce cell death in cerebral ischemia.[22] Thus, the intracellular signaling pathway mediated by NTs can act not only for survival but also as a proapoptotic or pronecrotic pathway in neuronal cells. Whether coronary BDNF induces cell necrosis or apoptosis of vascular cells warrants further investigation. BDNF enhanced the activity of NAD(P)H oxidase and the generation of superoxide in cultured smooth muscle cells. Because oxygen radicals activate matrix metalloproteinases,[23] the oxidative stress by BDNF might induce the instability of atherosclerotic plaques. On the other hand, BDNF has important roles in survival or as a development factor even in nonneuronal tissues such as endothelial cells.[24] BDNF in vasculature may work as a protective factor for endothelial cells in a counterregulatory mechanism. Further investigation is needed to clarify the precise mechanisms of BDNF in the pathogenesis of UAP.

In the present investigation, increased Cs-Ao differences in BDNF were observed in the UAP patents, although there was no significant difference in the numbers of diseased vessels or the degree of coronary stenosis between the SAP and UAP groups. These findings indicate that BDNF in the coronary circulation seems to influence the disease state of angina pectoris rather than the degree of coronary atherosclerosis and plaque formation. Acute coronary syndrome usually occurs at sites with <70% stenosis, as determined by angiographic studies performed in patients before the onset of coronary events. Therefore, BDNF might be involved in the vulnerability of atherosclerotic plaques. There are several possible origins of BDNF in the coronary circulation, including vascular smooth muscle cells, accumulating inflammatory cells, adventitial fibroblasts, cardiac myocytes, and neural cells. Recently, it has been reported that platelets release BDNF; therefore, activated platelets are a potential origin of BDNF.[25] However, the intense immunoreactivity in patients with UAP suggests that BDNF in the coronary circulation likely comes from atherosclerotic plaques. Plaque rupture and erosion are key events in the pathogenesis of acute coronary syndrome, including unstable angina; therefore, it is possible that disrupted atherosclerotic plaques release BDNF. Some other factors may be involved in the increased Cs-Ao differences in BDNF. Thus, further investigations are necessary to establish the causal relationship between BDNF and plaque rupture.

Psychological stress produces significant increases in heart rate and blood pressure, which might lead to an increased myocardial oxygen demand. Kario et al[26] demonstrated that earthquake-induced stress increased not only blood pressure and blood viscosity determinants but also fibrin turnover with endothelial cell stimulation in a group of hypertensive elderly subjects, suggesting that acute stress might trigger cardiovascular events. On the other hand, psychological stress increases the secretion of NTs from central and peripheral nerves.[27–29] Neuropsychological studies demonstrate that psychological stress such as immobilization stress increases BDNF mRNA expression in the hypothalamus of experimental models.[29] There is no direct evidence that BDNF in the coronary vasculature is regulated by psychological stress in

humans; however, psychological stress might increase the production of BDNF in coronary beds, which in turn augments regional oxidative stress via NAD(P)H oxidase. Further investigation is needed to clarify the regulation of coronary BDNF and the direct effect of psychological stress on NT levels in the coronary vasculature.

Plasma BDNF levels are decreased in patients with psychological disorders such as depression, and the level recovers with antidepressant drug treatment.[27,30] In our study, we did not enroll patients with mental disorders, and no patients had taken antidepressant drugs or tranquilizers. Although the psychological states of the patients were not examined, we do not consider that the Cs-Ao differences in BDNF were influenced by these factors.

In conclusion, plasma BDNF, but not NT-3, was increased in the coronary circulation in patients with UAP, and BDNF expression was enhanced in coronary arteries of UAP patients. BDNF increased NAD(P)H oxidase activity and superoxide production in human CASMC culture. Our observations suggest that the enhanced oxidative stress induced by BDNF has an important role in plaque instability.

References

1. Ogawa K, Tsuji I, Shiono K, Hisamichi S. Increased acute myocardial infarction mortality following the 1995 great Hanshin-Awaji earthquake in Japan. *Int J Epidemiol*. 2000;29:449–455.
2. Rozanski A, Blumenthal JA, Kaplan J. Impact of psychological factors on the pathogenesis of cardiovascular disease and implications for therapy. *Circulation*. 1999;99:2192–2217.
3. Barde YA, Edgar D, Thoenen H. Purification of a new neurotrophic factor from mammalian brain. *EMBO J*. 1982;1:549–553.
4. Hohn A, Leibrock J, Bailey K, Barde YA. Identification and characterization of a novel member of the nerve growth factor/brain-derived neurotrophic factor family. *Nature*. 1990;344:339–341.
5. Lewin GR, Barde YA. Physiology of the neurotrophins. *Annu Rev Neurosci*. 1996;19:289–317.
6. DiStefano PS, Friedman B, Radziejewski C, Alexander C, Boland P, Schick CM, Lindsay RM, Wiegand SJ. The neurotrophins BDNF, NT-3, and NGF display distinct patterns of retrograde axonal transport in peripheral and central neurons. *Neuron*. 1992;8:983–993.
7. Lessmann V, Gottmann K, Malcangio M. Neurotrophin secretion: current facts and future prospects. *Prog Neurobiol*. 2003;69:341–374.
8. Patapoutian A, Reichardt LF. Trk receptors: mediators of neurotrophin action. *Curr Opin Neurobiol*. 2001;11:272–280.
9. Roux PP, Barker PA. Neurotrophin signaling through the p75 neurotrophin receptor. *Prog Neurobiol*. 2002;67:203–233.
10. Clegg DO, Large TH, Bodary SC, Reichardt LF. Regulation of nerve growth factor mRNA levels in developing rat heart ventricle is not altered by sympathectomy *Dev Biol*. 1989;134:30–37.
11. Yamamoto M, Sobue G, Yamamoto K, Terao S, Mitsuma T. Expression of mRNAs for neurotrophic factors (NGF, BDNF, NT-3, and GDNF) and their receptors (p75NGFR, trkA, trkB, and trkC) in the adult human peripheral nervous system and nonneural tissues. *Neurochem Res*. 1996;21:929–938.
12. Donovan MJ, Miranda RC, Kraemer R, McCaffrey TA, Tessarollo L, Mahadeo D, Sharif S, Kaplan DR, Tsoulfas P, Parada L, et al. Neurotrophin and neurotrophin receptors in vascular smooth muscle cells: regulation of expression in response to injury. *Am J Pathol*. 1995;147:309–324.
13. Barouch R, Appel E, Kazimirsky G, Brodie C. Macrophages express neurotrophins and neurotrophin receptors: regulation of nitric oxide production by NT-3. *J Neuroimmunol*. 2001;112:72–77.
14. Kerschensteiner M, Gallmeier E, Behrens L, Leal VV, Misgeld T, Klinkert WE, Kolbeck R, Hoppe E, Oropeza-Wekerle RL, Bartke I, Stadelmann C, Lassmann H, Wekerle H, Hohlfeld R. Activated human T cells, B cells, and monocytes produce brain-derived neurotrophic factor in vitro and in inflammatory brain lesions: a neuroprotective role of inflammation? *J Exp Med*. 1999;189:865–870.
15. Nakahashi T, Fujimura H, Altar CA, Li J, Kambayashi J, Tandon NN, Sun B. Vascular endothelial cells synthesize and secrete brain-derived neurotrophic factor. *FEBS Lett*. 2000;470:113–117.
16. Azumi H, Inoue N, Ohashi Y, Terashima M, Mori T, Fujita H, Awano K, Kobayashi K, Maeda K, Hata K, Shinke T, Kobayashi S, Hirata K, Kawashima S, Itabe H, Hayashi Y, Imajoh-Ohmi S, Itoh H, Yokoyama M. Superoxide generation in directional coronary atherectomy specimens of patients with angina pectoris: important role of NAD(P)H oxidase. *Arterioscler Thromb Vasc Biol*. 2003;22:1838–1844.
17. Kim SH, Won SJ, Sohn S, Kwon HJ, Lee JY, Park JH, Gwag BJ. Brain-derived neurotrophic factor can act as a pronecrotic factor through transcriptional and translational activation of NADPH oxidase. *J Cell Biol*. 2002;159:821–831.
18. Azumi H, Inoue N, Takeshita S, Rikitake Y, Kawashima S, Hayashi Y, Itoh H, Yokoyama M. Expression of NADH/NADPH oxidase p22phox in human coronary arteries. *Circulation*. 1999;100:1494–1498.
19. Schabitz WR, Schwab S, Spranger M, Hacke W. Intraventricular brain-derived neurotrophic factor reduces infarct size after focal cerebral ischemia in rats. *J Cereb Blood Flow Metab*. 1997;17:500–506.
20. Huang BR, Gu JJ, Ming H, Lai DB, Zhou XF. Differential actions of neurotrophins on apoptosis mediated by the low affinity neurotrophin receptor p75NTR in immortalised neuronal cell lines. *Neurochem Int*. 2000;36:55–65.
21. Ishikawa Y, Ikeuchi T, Hatanaka H. Brain-derived neurotrophic factor accelerates nitric oxide donor-induced apoptosis of cultured cortical neurons. *J Neurochem*. 2000;75:494–502.
22. Bates B, Hirt L, Thomas SS, Akbarian S, Le D, Amin-Hanjani S, Whalen M, Jaenisch R, Moskowitz MA. Neurotrophin-3 promotes cell death induced in cerebral ischemia, oxygen-glucose deprivation, and oxidative stress: possible involvement of oxygen free radicals. *Neurobiol Dis*. 2002;9:24–37.
23. Inoue N, Takeshita S, Gao D, Ishida T, Kawashima S, Akita H, Tawa R, Sakurai H, Yokoyama M. Lysophosphatidylcholine increases the secretion of matrix metalloproteinase 2 through the activation of NADH/NADPH oxidase in cultured aortic endothelial cells. *Atherosclerosis*. 2001;155:45–52.
24. Donovan MJ, Lin MI, Wiegn P, Ringstedt T, Kraemer R, Hahn R, Wang S, Ibanez CF, Rafii S, Hempstead BL. Brain derived neurotrophic factor is an endothelial cell survival factor required for intramyocardial vessel stabilization. *Development*. 2000;127:4531–4540.
25. Fujimura H, Altar CA, Chen R, Nakamura T, Nakahashi T, Kambayashi J, Sun B, Tandon NN. Brain-derived neurotrophic factor is stored in human platelets and released by agonist stimulation. *Thromb Haemost*. 2002;87:728–734.
26. Kario K, Matsuo T, Kayaba K, Soukejima S, Kagamimori S, Shimada K. Earthquake-induced cardiovascular disease and related risk factors in focusing on the great Hanshin-Awaji earthquake. *J Epidemiol*. 1998;8:131–139.
27. Smith MA, Makino S, Altemus M, Michelson D, Hong SK, Kvetnansky R, Post RM. Stress and antidepressants differentially regulate neurotrophin 3 mRNA expression in the locus coeruleus. *Proc Natl Acad Sci U S A*. 1995;92:8788–8792.
28. Russo-Neustadt A. Brain-derived neurotrophic factor, behavior, and new directions for the treatment of mental disorders. *Semin Clin Neuropsychiatry*. 2003;8:109–118.
29. Rage F, Givalois L, Marmigere F, Tapia-Arancibia L, Arancibia S. Immobilization stress rapidly modulates BDNF mRNA expression in the hypothalamus of adult male rats. *Neuroscience*. 2002;112:309–318.
30. Shimizu E, Hashimoto K, Okamura N, Koike K, Komatsu N, Kumakiri C, Nakazato M, Watanabe H, Shinoda N, Okada S, Iyo M. Alterations of serum levels of BDNF in depressed patients with or without antidepressants. *Bio Psychiatry*. 2003;54:70–75.

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