Journal of the American College of Cardiology
© 2000 by the American College of Cardiology
Published by Elsevier Science Inc.

Vol. 36, No. 3, 2000
ISSN 0735-1097/00/$20.00
PII S0735-1097(00)00778-6

Increased Expression of Peripheral Benzodiazepine Receptors on Leukocytes in Silent Myocardial Ischemia

Antonino Mazzone, MD,* Iolanda Mazzucchelli, BSc,* Monia Vezzoli, MD,* Elena Ottini, MD,* Carla Auguadro, MD,† Alessandra Serio, MD,† Colomba Falcone, MD†

Pavia, Italy

OBJECTIVES The purpose of this study was to evaluate benzodiazepine receptor expression on leukocytes from patients with symptomatic or silent myocardial ischemia.

BACKGROUND Silent myocardial ischemia is frequently observed in patients with coronary artery disease. Pain can be effectively controlled by various endogenous mechanisms. Benzodiazepines and their receptors play key roles in pain, in interactions with peptide opioids, in inflammation and in the response to stress.

METHODS The study group consisted of 57 patients with reproducible exercise-induced myocardial ischemia. The presence of a constant behavior in the anginal pain perception during both exercise-induced ischemia and daily life was the most important inclusion criterion. Venous blood samples were taken from all patients to evaluate the expression of peripheral benzodiazepine receptors by flow cytometry. The study cohort was classified into two groups: 24 patients who had anginal pain both at home and during the exercise stress test and 33 patients who were asymptomatic during both daily life and exercise-induced ischemia.

RESULTS Flow cytometry analysis showed increased expression of peripheral benzodiazepine receptors on all types of leukocytes in the asymptomatic patients. The difference was statistically significant for lymphocytes ($p < 0.005$), monocytes ($p < 0.001$) and granulocytes ($p < 0.001$).

CONCLUSIONS These data show that expression of peripheral benzodiazepine receptors was higher in patients with silent myocardial ischemia than in symptomatic patients. (J Am Coll Cardiol 2000;36: 746–50) © 2000 by the American College of Cardiology

Coronary artery disease (CAD) is a progressive atherosclerotic process (1) that results in a spectrum of clinical manifestations ranging from asymptomatic and stable angina to the acute coronary syndromes, unstable angina, acute myocardial infarction (MI) and sudden ischemic death (2). The absence of anginal pain during transitory episodes of myocardial ischemia occurring during both daily life (3,4) and stress tests is frequent (5,6); similarly, acute ischemia during MI and during angioplasty-induced coronary occlusion may not be associated with anginal pain (7,8). The mechanisms responsible for silent ischemia are not well understood, and many theories have been advanced (4). The presence or absence of angina may be partly explained by individual differences in the pain threshold (5,6). A generalized hyposensitivity to different painful stimuli and a higher pain threshold have been reported in patients with silent ischemia compared with symptomatic patients (4–6).

The endogenous opioid system plays an important role in pain modulation (7). An increase in beta-endorphin plasma levels, coinciding with an increase in plasma cortisol, has been documented during exercise as part of the physiologic stress response (3,8). Benzodiazepines could antagonize opioid-induced analgesia by enhancing the action of gamma-aminobutyric acid (GABA) on $GABA_a$ receptors in the pain modulation circuits (9). Some evidence connects peripheral benzodiazepine receptors (PBRs) with voltage-dependent calcium channel antagonists in relaxing smooth muscle and inhibiting beta-endorphin release in a cultured pituitary cell line (10). In addition, various investigators have shown that benzodiazepine treatment improves control of angina (11) and that PBRs could play a role in the stress response (12), in the interactions of the opioid system (7) and in steroid biosynthesis (13). Benzodiazepines have been shown to interact with opioid antinociception (9). Furthermore, in the last few years, the roles of inflammation and leukocytes in CAD have been underlined (14,15). In peripheral ischemic and inflamed tissue (16), an interaction between immune cell–derived opioids and other receptors localized on sensory nerve terminals can result in strong, clinically measurable analgesia (17–19). Given that PBRs affect opioid-induced antinociception and modulation of pain in animals, we hypothesized that the expression of PBRs on leukocytes is different in patients with angina than in patients without anginal symptoms during myocardial ischemia. We tested this hypothesis by measuring PBRs expression using flow cytometry on various sets of leukocytes in these two groups of patients.

From the *Department of Internal Medicine and Nephrology and †Department of Cardiology, IRCCS, San Matteo Hospital, University of Pavia, Pavia, Italy. This study was supported by Grant 96/03, current reserach at IRCCS, San Matteo Hospital, Pavia, Italy.

Manuscript received August 29, 1999; revised manuscript received March 1, 2000, accepted April 13, 2000.

Abbreviations and Acronyms

CAD = coronary artery disease
DBI = diazepam-binding inhibitor
ECG = electrocardiogram or electrocardiographic
EST = ergometric stress test
GABA = γ-aminobutyric acid
MI = myocardial infarction
PBRs = peripheral benzodiazepine receptors
PBS = phosphate-buffered saline

METHODS

Patient selection. Our study group consisted of 57 men, 24 of whom reported anginal pain both at home and during two exercise stress tests (group P) and 33 of whom were asymptomatic during both daily life and exercise-induced ischemia (group A). The inclusion criteria were evidence of myocardial ischemia during these two exercise stress tests and reproducibility of the same original pain perception or lack of during both exercise-induced ischemia and daily life (patients always symptomatic or patients always asymptomatic). The patients studied were male because the reproducibility of pain perception in women can be confounded by the effects of neurohormonal changes. Patients with intercurrent inflammatory conditions, as well as patients taking nonsteroidal anti-inflammatory drugs, steroids, benzodiazepine or antiepileptic agents were excluded from the study. All tests were performed in pharmacologic washout; calcium channel blocking agents and nitrates were suspended 48 h before. No patient was receiving beta-blocker therapy at the time of the study. Beta-blockers were gradually reduced and stopped one week before our examination. No patient received digitalis. Informed, written consent for all tests was obtained from all subjects. The study was approved by the Ethical Committee of our hospital.

Exercise stress testing. A multistage bicycle ergometric stress test (EST) was performed in all patients. The initial work load was 25 W, and the subsequent stepwise increments were 25 W every 2 min at a pedalling frequency of 60 rpm. A standard 12-lead electrocardiogram (ECG) and blood pressure were recorded in basal conditions, with the patient lying in a supine position for 10 min and then just before the test, at the end of each stage, at the appearance of ECG signs of ischemia or anginal pain, at peak exercise and every 3 min during recovery. Three ECG leads (D_3, V_5, V_6) were continuously monitored and displayed on an oscilloscope throughout the test. During the test, the patients were continuously asked about the onset of angina or other symptoms. A *positive ECG response* was defined as the occurrence of at least 1 mm ST segment depression, as compared with the baseline tracing. The *ischemic threshold* was defined as the appearance of 1 mm flat or downsloping ST segment depression 0.08 s after the J point; the *angina threshold* was defined as the time of the first report of chest pain during the test. EST was stopped when moderate to severe angina, dyspnea, muscle fatigue, ST segment depression >3 mm or major arrhythmias occurred. The rate–pressure product (RPP: heart rate × systolic blood pressure) was calculated in basal conditions, at the ischemic threshold and at peak exercise.

Coronary arteriography. All patients underwent cardiac ventriculography and Sones selective coronary arteriography within 7 ± 5 days after the exercise stress test. Left ventriculography was performed before coronary arteriography in the 30° right anterior oblique projection; ventricular volumes and left ventricular ejection fraction were calculated by the standard area–length method. *Significant CAD* was defined as ≥50% narrowing of the lumen of at least one of the three main arteries or their major branches.

Flow cytometry analysis. Expression of PBRs was measured by direct immunofluorescence methods using a fluorescent probe (Bodipy Ro-1986, Molecular Probes, Eugene, Oregon), a fluorescence derived from desdiethylfluorazepam with a high affinity and selectivity for benzodiazepine receptors. Venous blood samples were taken from all patients at 8 AM, before the first exercise stress test, in reproducible and standardized conditions. The analysis of PBRs expression on the plasma membrane of the leukocytes was performed as described.

A sample of 100 μl of Na_2 EDTA preserved blood was treated with 2 ml of ice-cold erythrocyte-lysing solution (NH_4Cl 0.208 g; Na_2EDTA 0.1 mg; $KHCO_3$ 0.025 g in 250 ml H_2O), resuspended in 100 μl of cold phosphate-buffered saline (PBS) (NaCl 8 g/liter; KCl 0.2 g/liter; Na_2HPO_4, 2.89 g/liter; KH_2PO_4 0.23 g/liter; BSA 2 g/liter; NaN_3 0.2 g/liter) and incubated for 30 min at 0° to 5°C in the dark with 10 μl of Bodipy Ro-1986 at 5×10^{-5} mol/liter.

After incubation, cells were washed three times and then resuspended in 500 μl of cold PBS containing NaN_3 and analyzed by flow cytometry (Coulter Epics Profile II). Gating for lymphocytes, monocytes and granulocytes was determined by the dot blot generated by the forward angle scatter versus right angle scatter; 10,000 gated cells per sample were analyzed, and the fluorescence was recorded as a mean channel number over the logarithmic range of 1 to 1,024. The PBRs are expressed on the plasma membrane and on the mitochondrial membrane (20); in our study, we evaluated the plasma membrane sites of PBRs. The expression of membrane receptors was reported as sites per cell as described by Caranyon et al. (21).

Binding specificity assay. To verify the fluorescence specificity, the agonist fluorazepam (without fluorescein) or the agonist Ro5-4684 (Sigma, St. Louis, Missouri) or the antagonist PK-11195 (Sigma) was added to our prelabeled samples. In short, 100 μl of prelabeled cells was incubated for 30 min at 37°C with 10 μl of fluorazepam 10^{-4} mol/liter or Ro5-4684 3×10^{-4} mol/liter or PK-11195 3×10^{-4} mol/liter. After incubation, cells were washed three times with cold PBS and then resuspended in 250 μl of cold PBS containing NaN_3. Samples were analyzed by flow cytometry under the same conditions as samples stained with Bodipy Ro-1986.

Table 1. Clinical, Angiographic and Ergometric Features of Symptomatic (Group P) and Asymptomatic (Group A) Patients During Myocardial Ischemia

	Group P (n = 24)	Group A (n = 33)	p Value
Clinical features			
Age (years)	60.7 ± 8.6	64.9 ± 9.4	NS
Arterial hypertension	4	6	NS
Hyperlipidemia	10	14	NS
Diabetes mellitus	3	4	NS
Smoking	9	12	NS
Coronary angiography			
Single-vessel disease	10	7	NS
Multivessel disease	14	26	NS
Ejection fraction	58.1 ± 10.2	60.1 ± 9.4	NS
LVEDV (ml)	115 ± 26	124 ± 21	NS
LVEDP (mm Hg)	16.4 ± 8.1	14.6 ± 3.9	NS
EST			
Baseline RPP (beats/min × mm Hg)	9.243 ± 2.232	9.623 ± 2.056	NS
IT-RPP (beats/min × mm Hg)	19.978 ± 6.723	20.121 ± 5.734	NS
Peak RPP (beats/min × mm Hg)	21.043 ± 4.585	22.347 ± 6.573	NS
Peak ST segment depression (mm)	1.92 ± 1.51	2.24 ± 1.82	NS

Data are presented as the mean value ± SD or number of patients.
EST = exercise stress test; IT = ischemic threshold; LVEDP and LVEDV = left ventricular end-diastolic pressure and volume, respectively; RPP = rate–pressure product.

Statistical analysis. Results are expressed as the mean value ± SD. Mann-Whitney *U* nonparametric analysis was used to investigate differences between the two groups. The Stat View program for Apple Computers was used, and $p < 0.05$ was considered statistically significant.

RESULTS

Our study group consisted of 57 men (mean age 62.5 ± 8.6 years) classified into two groups: 24 patients (with a history of angina pectoris during daily life (group P: 9 patients with effort angina, 4 patients with angina at rest and 11 patients with mixed angina) and 33 patients with silent ischemia documented during the exercise stress test (group A), including 17 patients who had had nonpainful ischemic episodes during domiciliary Holter recordings. The other clinical characteristics of the two groups of patients are reported in Table 1. There were no statistically significant differences in age, ECG-documented severity of angina, hypertension, hyperlipidemia, diabetes or smoking. All patients in group P reported anginal pain during exercise; moderate to severe dyspnea was experienced during EST in group A patients. The entity of ST segment depression at peak exercise was similar between the two groups.

Patients with silent ischemia had greater expression of PBRs on leukocytes than did patients reporting angina pectoris. The binding specificity of PBRs was evaluated using a high concentration of the agonist Ro5-4684 (Fig. 1).

On lymphocytes, the PBRs expression in group A was $2.0 \pm 0.4 \times 10^5$ sites/cell, and in group P it was $1.2 \pm 0.4 \times 10^5$ sites/cell ($p < 0.005$). On monocytes, the PBRs expression in Group A was $13.4 \pm 0.9 \times 10^5$ sites/cell, and in group P it was $8.0 \pm 0.5 \times 10^5$ sites/cell ($p < 0.001$). On granulocytes, the PBRs expression in group A was $7.22 \pm 0.8 \times 10^5$ sites/cell, and in group P it was $4.25 \pm 0.9 \times 10^5$ sites/cell ($p < 0.001$) (Fig. 2).

DISCUSSION

Our data show that the expression of PBRs on leukocytes from patients with documented ischemia is significantly greater in patients with silent ischemia than in patients with symptomatic ischemia.

The PBRs are expressed on all human peripheral blood leukocyte subsets, but mainly on monocytes and granulocytes, whereas natural killer cells and B- and T-lymphocytes display lower levels of PBRs (20). The PBRs have been

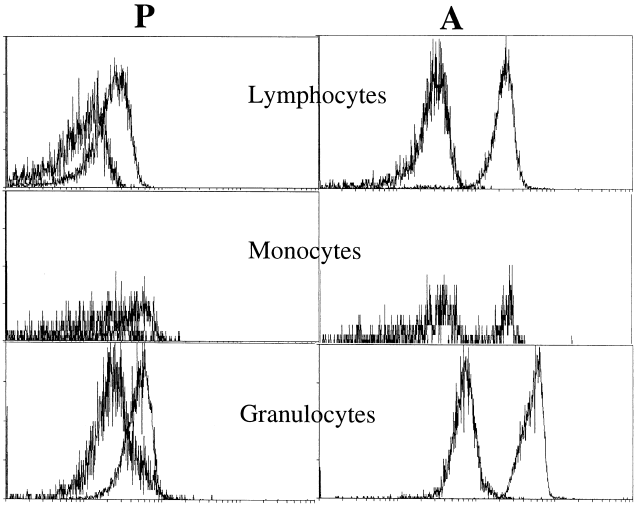

Figure 1. The fluorescence intensity of Bodipy Ro-1986 in a symptomatic patient and in an asymptomatic patient before and after incubation with a high concentration of the agonist Ro5-4864 without fluorescein. Specific staining was expressed as the mean channel fluorescence intensity.

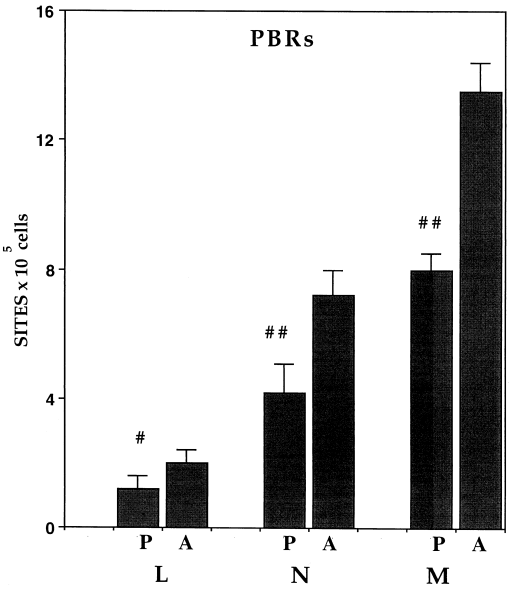

Figure 2. In patients with silent ischemia (A), the PBRs expression was significantly higher than that in symptomatic patients (P) in all types of leukocytes. L = lymphocytes; M = monocytes; N = neutrophils. #$p < 0.005$. ##$p < 0.001$.

found to modulate immunocyte functions such as chemotaxis (22), respiratory burst (21) and cytokine secretion (23). Modulation of chemotaxis limits the spread of chemotactic agents and preserves cells from free radical damage (21).

Various endogenous substances, such as diazepam-binding inhibitor (DBI), are able to bind to PBRs; these substances have been found in human blood and urine and are involved in pain control (7,9,12,13). The interaction between PBRs and morphine-induced antinociception is abolished by benzodiazepine antagonists such as flumazenil (9,13), indicating, again, that PBRs are involved in pain control. Opioid receptors on peripheral sensory nerves are upregulated during inflammation. Opioid receptors are expressed on immune cells and can modulate the behavior and release of cytokines, inducing local analgesia (7). Moreover, neuroactive substances and related receptors could inhibit nociception of the compromised heart by directly influencing and modulating the microenvironment (4,7,19).

The absence of pain in certain patients with myocardial ischemia could be linked to an increased expression of PBRs. The possible functional significance of the increased number of benzodiazepine-binding sites on the plasma membrane could be related to DBI, which enhances the production of interleukin-1 and tumor necrosis factor (24); these cytokines are able to increase the expression of selectins, which have a key role in inflammation-induced pain (17). A balanced control of pain with PBRs and opioids may take place through cytokine release.

It is interesting that in epileptic patients, increased DBI and decreased PBRs are related to increased excitability and resistance to specific therapy (25), whereas increased PBRs expression on all leukocyte subsets is related to a decrease of DBI, which raises the pain threshold (4,6,12,13). Thus, the concentrations of DBI, opioids and benzodiazepines are fundamental in the control of pain responses and threshold of pain. The PBRs may be a critical element in the interrelation between brain, behavior, immunity and modulation of pain circuits.

Study limitations. Our study has some limitations. Only one determination at a single time point was made, but the samples were analyzed in duplicate. Different endogenous substances capable of binding to PBRs have been found in extracts of various rat organs and in human blood and urine, and a benzodiazepine receptor–mediated model of pain has also been advanced. We did not test DBI, benzodiazepines or endorphins in our patients. In this study, we did not measure the algogenic stimulus in myocardial ischemia, but we have studied this in a previous report (5).

Conclusions. Increased or decreased PBRs density may reflect endogenenous ligand activity or regulatory processes such as DBI or endogenous benzodiazepines. Modifications of the concentration of DBI or endogenous benzodiazepines in silent myocardial ischemia could explain the observed PBRs upregulation. The higher density of PBRs on leukocytes of patients with asymptomatic ischemia is related to qualitative differences in central and/or peripheral pain mechanism regulation. Further studies are needed to explain whether the origin of these different expressions of PBRs are genetically determined or secondary to the stressful events and neurohormonal response.

Reprint requests and correspondence: Dr. Antonino Mazzone, Adjunct Professor of Immunopharmacology, Department of Internal Medicine and Nephrology, University of Pavia, IRCCS, San Matteo Hospital, P.le Golgi 2, 27100 Pavia, Italy. E-mail: a.mazzone@smatteo.pv.it.

REFERENCES

1. Ross R. Mechanisms of disease: atherosclerosis—an inflammatory disease. N Engl J Med 1999;340:115–26.
2. Shah PK. New insights into the pathogenesis and prevention of acute coronary syndromes. Am J Cardiol 1997;79:17–23.
3. Falcone C, Specchia G, Rondanelli R, et al. Correlation between beta-endorphin plasma levels and anginal symptoms in patients with coronary artery disease. J Am Coll Cardiol 1998;11:719–23.
4. Meller ST, Gebhart GF. Silent ischemia: a hypothetical mechanism. Neurosci Biobehav Rev 1993;17:229–36.
5. Falcone C, Sconocchia R, Guasti L, Codega S, Montemartini C, Specchia G. Dental pain threshold and angina pectoris in patients with coronary artery disease. J Am Coll Cardiol 1998;12:348–52.
6. Glazier JJ, Chierchia S, Brown MJ, Maseri A. The importance of generalized defective perception of painful stimuli as a cause of silent myocardial ischemia in chronic stable angina pectoris. Am J Cardiol 1986;58:667–72.
7. Stein C. The control of pain in peripheral tissue by opioids. N Engl J Med 1995;332:1685–90.
8. Falcone C, Guasti L, Ochan M, et al. Beta-endorphins during coronary angioplasty in patients with silent or symptomatic myocardial ischemia. J Am Coll Cardiol 1993;22:1614–20.
9. Gear RW, Miaskowski C, Heller PH, Paul SM, Gordon NC, Levine

JD. Benzodiazepine mediated antagonism of opioid analgesia. Pain 1997;71:25–29.
10. Bisserbe JC, Patel J, Eskay RL. Evidence that the peripheral-type benzodiazepine receptor ligand Ro5-4864 inhibits β-endorphin release from AJT-20 cells by blockage of voltage-dependent calcium channels. J Neurochem 1986;47:1419–24.
11. Williams RB. Do benzodiazepines have a role in the prevention or treatment of coronary heart disease and other major medical disorders? J Psych Res 1990;24:51–6.
12. Drugan RC, Holmes PV. Central and peripheral benzodiazepine receptors: involvement in an organism's response to physical and psychological stress. Neurosci Behav Rev 1991;15:277–98.
13. Papadopoulos V, Nowzari FB, Kruger KE. Hormone-stimulated steroidogenesis is coupled to mitochondrial benzodiazepine receptors: tropic hormone action on steroid biosynthesis is inhibited by flunitrazepam. J Biol Chem 1991;266:3682–7.
14. Mazzone A, De Servi S, Ricevuti G, et al. Increased expression of neutrophil and monocyte adhesion molecules in unstable coronary artery disease. Circulation 1993;88:358–63.
15. De Servi S, Mazzone A, Ricevuti G, et al. Clinical and angiographic correlates of leukocyte activation in unstable angina. J Am Coll Cardiol 1995;26:1146–50.
16. Mazzone A, De Servi S, Mazzucchelli I, et al. Increased expression of CD11b/CD18 on phagocytes in ischaemic disease: a bridge between inflammation and coagulation. Eur J Clin Invest 1997;27:648–52.
17. Machelska H, Cabot PJ, Mousa SA, Zhang Q, Stein C. Pain control in inflammation governed by selectins. Nature Med 1998;4:1425–8.
18. Porreca F, Lai J, Malan TP. Can inflammation relieve pain? Nature Med 1998;4:1359–60.
19. Stefano GB, Salzet B, Fricchione GL. Enkelytin and opioid peptide association in invertebrates and vertebrates: immune activation and pain. Immunol Today 1998;19:265–9.
20. Cahard D, Canat X, Carayon P, Roque C, Casellas P, Le Fur G. Subcellular localization of peripheral benzodiazepine receptors on human leukocytes. Lab Invest 1994;70:23–8.
21. Caranyon P, Portier M, Dussossoy D, et al. Involvement of peripheral benzodiazepine receptors in the protection of hematopoietic cells against oxygen radical damage. Blood 1996;87:3170–8.
22. Ruff MR, Pert CB, Weber RJ, Wahl LM, Wahl SM, Paul SM. Benzodiazepine receptor–mediated chemotaxis of human monocytes. Science 1985;229:1281–5.
23. Poole S, Cunha FQ, Selkirk S, Lorenzetti BB, Ferreira SH. Cytokine-mediated inflammatory hyperalgesia limited by interleukin-10. Br J Pharmacol 1995;115:684–8.
24. Taupin V, Gogusev J, Descamps-Latscha B, Zavala F. Modulation of tumor necrosis factor-α, interleukin-1b, interleukin-6, interleukin-8 and granulocyte/macrophage colony-stimulating factor expression in human monocytes by an endogenous anxiogenic benzodiazepine ligand, triakontatetraneuropeptide: evidence for a role of prostaglandins. Mol Pharmacol 1993;43:64–9.
25. Caldiroli E, Marino F, Cosentino M, et al. Peripheral benzodiazepine receptor expression on leukocytes and neutrophil function during anticonvulsant monotherapy. Pharmacology 1998;57:215–21.