Inverse alterations of BCKA dehydrogenase activity in cardiac and skeletal muscles of diabetic rats

YOLANDA B. LOMBARDO, CYNTIA SERDIKOFF, MANIKKAVASAGAR THAMOTHARAN, HARBHajan S. PAUL, AND SIAMAK A. ADIBI
Clinical Nutrition Research Unit, Department of Medicine, University of Pittsburgh
School of Medicine, Pittsburgh, Pennsylvania 15213

Lombardo, Yolanda B., Cynthia Serdikoff, Manikkavasagar Thamotharan, Harbhajan S. Paul, and Siamak A. Adibi. Inverse alterations of BCKA dehydrogenase activity in cardiac and skeletal muscles of diabetic rats. Am. J. Physiol. 277 (Endocrinol. Metab. 40): E685–E692, 1999.—Rat cardiac and skeletal muscles, which have been used as model tissues for studies of regulation of branched-chain α-keto acid (BCKA) oxidation, vary greatly in the activity state of their BCKA dehydrogenase. In the present experiment, we have investigated whether they also vary in response of their BCKA dehydrogenase to a metabolic alteration such as diabetes and, if so, to investigate the mechanism that underlies the difference. Diabetes was produced by depriving streptozotocin-treated rats of insulin administration for 96 h. The investigation of BCKA dehydrogenase in the skeletal muscle (gastrocnemius) showed that diabetes 1) increased its activity, 2) increased the protein and gene expressions of all of its subunits (Eα, Eβ, Eγ), 3) increased its activity state, 4) decreased the rate of its inactivation, and 5) decreased the protein expression of its associated kinase (BCKAD kinase) without affecting its gene expression. In sharp contrast, the investigation of BCKA dehydrogenase in the cardiac muscle showed that diabetes 1) decreased its activity, 2) had no effect on either protein or gene expression of any of its subunits, 3) decreased its activity state, 4) increased its rate of inactivation, and 5) decreased both the protein and gene expressions of its associated kinase. In conclusion, our data suggest that, in diabetes, the protein expression of BCKAD kinase is downregulated posttranscriptionally in the skeletal muscle, whereas it is upregulated pretranslationally in the cardiac muscle, causing inverse alterations of BCKA dehydrogenase activity in these muscles.

MUSCLES PLAY A MAJOR ROLE IN oxidation of branched-chain amino acids (2). These amino acids (leucine, isoleucine, and valine) combined are the major constituents of dietary and body proteins. Transamination of these amino acids results in production of branched-chain keto acids (BCKA). The key enzyme regulating oxidation of these keto acids is BCKA dehydrogenase. It exists in interconvertible phosphorylated (inactive) and dephosphorylated (active) forms (32).

Previous studies from our laboratory (27, 28, 31) and others (3, 11, 26) have shown that the activity of BCKA dehydrogenase in muscles is greatly altered in metabolic disorders such as diabetes. However, there have been very few studies on the mechanism of these alterations in the muscle. The only mechanism that has been implicated is changes in the activity state (proportion of active to inactive form) of the enzyme (3, 11, 26, 31). On the other hand, studies in the liver have shown that there are other mechanisms responsible for alteration of BCKA dehydrogenase activity. For example, Chico et al. (7) found that dexamethasone and AMP treatment increases the amount of BCKA dehydrogenase in primary cultured hepatocytes by increasing its gene expression without a significant change in the activity state of BCKA dehydrogenase. As yet, there has not been any study on the effect of diabetes on molecular expressions of any component of BCKA dehydrogenase complex in muscles. Therefore, an aim of the present study was to fill this gap in the current knowledge.

There is another compelling reason for investigating the mechanisms of alterations of BCKA dehydrogenase activity in muscles. Although both the cardiac and skeletal muscles have been used for studies of regulation of muscle BCKA oxidation (5, 13, 14), they differ greatly in the activity state of their BCKA dehydrogenase. The enzyme is substantially active in the cardiac muscle (11, 26), whereas it is almost totally inactive in the resting skeletal muscle (3, 33). This raises the possibility that these muscles respond differently to metabolic alterations. Therefore, another aim of the present study was to investigate this possibility by determining whether diabetes has the same or different effects on BCKA dehydrogenase activity in the cardiac and skeletal muscles and to determine the mechanisms underlying these effects. These studies were performed in the muscles of streptozotocin-treated rats deprived of insulin administration for 96 h before experiments.

MATERIALS AND METHODS

Animals and their treatment. Twenty rats (male Sprague-Dawley, 200–250 g) were made diabetic and another twenty served as the control group. Rats were housed in individual cages in air-conditioned quarters with a controlled 12:12-h light-dark cycle and received powdered Purina Laboratory Chow and drinking water ad libitum. Diabetes was induced by intraperitoneal injection of streptozotocin (85 mg/kg body weight) in 0.05 M citrate-buffered saline, pH 4.5. Control rats were given a citrate-buffered saline injection. Induction of diabetes was assessed by estimation of blood glucose. Blood samples were collected in heparinized tubes and immediately centrifuged. Plasma was separated, and blood glucose was determined by the glucose oxidase method (43). Diabetic rats (>2 g glucose/l) received a daily injection of insulin (Humulin U Ultralente; Eli Lilly, Indianapolis, IN), 2–3 U/day for 8–10 days. At the end of this period, insulin therapy was...
withdrawn, and all the rats were euthanized ~96 h after the last insulin injection. During all periods, dietary treatment was maintained as described above. This protocol was similar to the one we previously used for studies of leucine oxidation and BCKA dehydrogenase in diabetic rats (22, 27, 28, 31).

Assay of BCKA dehydrogenase activity. The gastrocnemius and cardiac muscles were freeze-clamped with Wollenberger clamps precooled in liquid nitrogen. BCKA dehydrogenase complex was extracted from frozen tissues by a polyethylene glycol (PEG) precipitation method, as described previously (33). The activity of BCKA dehydrogenase in the cardiac muscle was determined spectrophotometrically by measuring the reduction of NAD\(^{+}\) (7). The complete assay mixture contained (final volume 1.5 ml) 30 mM potassium phosphate buffer (pH 7.4), 3 mM NAD\(^{+}\), 0.4 mM CoASH, 0.4 mM thiamine pyrophosphate, 2 mM dithiothreitol (DTT), 5 mM MgCl\(_2\), 10 units of pig heart dihydrolipoyl dehydrogenase, 0.1% vol/vol Triton X-100, 0.5 mM \(\alpha\)-ketosovalerate, and BCKA dehydrogenase complex (1.0–2.0 mg protein). All assays were performed at 30°C, and enzyme activity is expressed as nanomoles of NADH formed per minute per milligram of protein. The selection of the 30°C temperature was to maintain a linear rate of enzyme activity over the time course of the reaction (5–6 min).

This technique could not be used for measuring BCKA dehydrogenase activity in the skeletal muscle because the activity is much lower in the skeletal than in the cardiac muscle. We therefore used a radiochemical technique to measure BCKA dehydrogenase activity in the skeletal muscle. This did not present a problem with the objective of the study, which was to compare the activity between control and treated animals. The BCKA dehydrogenase activity in the skeletal muscle was determined by measuring the release of \(^{14}\)C from \(\alpha\)-ketol-[\(\text{\textsuperscript{14}C}\)]isocaproate (30). The \(\alpha\)-ketol-[\(\text{\textsuperscript{14}C}\)]isocaproate was prepared in this laboratory from \(\text{\textsuperscript{14}C}\)leucine (Amersham) by incubating it with \(\text{\textsuperscript{14}C}\)-labeled substrates for BCKA dehydrogenase (0.5–1.5 mg of protein). All assays were done in duplicate at 37°C, and the reaction was carried out for 15 min. At the end of this period, the reactions were stopped with 5 N H\(_2\)SO\(_4\), and \(^{14}\)CO\(_2\) was collected in hydroxide of Hyamine, and radioactivity was determined by liquid scintillation spectrometry (30).

Western blot analysis. BCKA dehydrogenase and BCKAD kinase were quantitatively precipitated by the PEG method, as described previously (33, 34). Identical amounts of proteins (100 µg), precipitated from the skeletal and cardiac muscles of control and diabetic rats, were suspended in SDS buffer [4% (wt/vol) SDS, 0.125 M Tris-HCl, pH 6.8, 20% (vol/vol) glycerol, and 0.125% (wt/vol) DTT] and boiled for 90 s. Samples were subjected to SDS-10% PAGE in the system of Laemmli (19). Resolved proteins were transferred onto nitrocellulose membranes [Hybond enhanced chemiluminescence (ECL); Amersham, Arlington Heights, IL] and subjected to immunoblot analysis. The membranes were incubated with either polyclonal antibody (1:2,000) raised against purified BCKA dehydrogenase complex or polyclonal antibody (1:500) raised against purified BCKAD kinase. Both antibodies were raised in rabbits by Pel Freez Biologicals (Rogers, AR). The membranes were then washed and incubated with a second antibody (peroxidase-conjugated goat anti-rabbit IgG) 1:2,000 for BCKA dehydrogenase or 1:1,000 for BCKAD kinase, as described previously (7, 34). Subunits of BCKA dehydrogenase and BCKAD kinase were detected with the ECL Western blotting system (Amersham). The intensity of bands was quantified by densitometry using Image software (Scion, Frederick, MD). RNA level for each sample was normalized to the abundance of \(\beta\)-actin RNA (CDNA clone; Clontech Laboratories, Palo Alto, CA), which served as an internal control for minor variations in sample loading.

Analysis of rate inactivation of BCKA dehydrogenase. BCKA dehydrogenase kinase (BCKAD kinase) complex was extracted from frozen cardiac (34) and skeletal (33) muscles. The rate of inactivation of BCKA dehydrogenase in the diabetic rats was extracted by RNAzol method (Tel-Test, Friendswood, TX). RNA (20 µg for BCKA dehydrogenase and 25 µg for BCKAD kinase) was fractionated on 0.9% wt/vol agarose gel containing formaldehyde and was blotted onto a Nytran membrane (Schleider and Schuell, Dassel, Germany). The membranes were hybridized as described previously (25). Cloned cDNAs encoding the E\(_{\alpha}\), E\(_{\beta}\), and E\(_{2}\) subunits of rat BCKA dehydrogenase were kindly provided by Dr. Robert Harris, Indiana University School of Medicine. Kinase cDNA was prepared using standard RT-PCR protocol. The primer sequences were as follows: CAGCCACCTTCTGAAAATG (sense primer corresponding to nucleotides 162–181) and CCTCAGCTAACAGGGTTACC (antisense primer corresponding to nucleotides 405–424). \(^{32}\)P-labeled cDNA probes were made by random primer technique (8) ([\(^{32}\)P]dCTP; Du Pont, NEN; Boston, MA; kit for radiolabeling DNA; Pharmacia Biotech, Piscataway, N J ). Blots were subjected to autoradiography with Kodak Biomax MS film at ~70°C for 72 h. The intensity of bands was quantified by densitometry using Image software (Scion, Frederick, MD). RNA level for each sample was normalized to the abundance of \(\beta\)-actin RNA (CDNA clone; Clontech Laboratories, Palo Alto, CA), which served as an internal control for minor variations in sample loading.

Statistics. All data are presented as means ± SE. Student’s t-test was used for statistical analysis of data, with the
corresponding P values given in figure legends. Each measurement was based on studies in 4–8 animals.

RESULTS

Diabetic status. After 96 h of insulin withdrawal, the blood glucose concentration of streptozotocin-treated rats was 336 ± 16 mg/dl. The blood glucose concentration of control rats was 128 ± 3 mg/dl. After 8–10 days of insulin therapy, the diabetic rats fully regained the weight they had lost earlier after the streptozotocin injection. However, again during the period of insulin withdrawal, they sustained some weight loss (20 ± 1 g).

BCKA dehydrogenase activity. The first step in our experiment was to compare the effect of diabetes on BCKA dehydrogenase activity in the skeletal (gastrocnemius) and cardiac muscles of the same animals. The results (means ± SE in 6–8 rats) showed that diabetes increased BCKA dehydrogenase activity by fourfold in the skeletal muscle (1.5 ± 0.2 vs. 6.1 ± 1.2 nmol × 10²·mg protein⁻¹·min⁻¹, P < 0.01) but that it decreased this activity by twofold in the cardiac muscle (740 ± 105 vs. 370 ± 24 nmol × 10²·mg protein⁻¹·min⁻¹, P < 0.01). We used the gastrocnemius muscle for our study because it contains both red and white fibers. Furthermore, it has been commonly used for studies of BCKA dehydrogenase activity because it is a validated representative of skeletal muscles (18).

BCKA dehydrogenase activity is a function of protein mass and the activity state of the enzyme in the tissues. The following studies were performed to determine which of these factors was responsible for the inverse alterations in diabetes.

Effect of diabetes on protein and gene expressions of BCKA dehydrogenase. BCKA dehydrogenase is composed of three catalytic proteins, designated as E₁, E₂, and E₃. The E₁ component is further composed of α-(E₁α) and β-(E₁β) subunits. The E₁ and E₂ components are specific for BCKA dehydrogenase, whereas E₃ is common to other dehydrogenases. Therefore, the present study includes the effect of diabetes on the protein expression of E₁α, E₁β, and E₂ subunits in the two muscle tissues.

Qualitative and quantitative analyses of Western blots showed that the amount of each subunit was significantly increased in the skeletal muscle of diabetic rats (Fig. 1). The increase was greater for E₁β (over 2-fold) than for either E₁α or E₂. In contrast to the skeletal muscle, diabetes had no significant effect on the amount of any subunit of BCKA dehydrogenase in the cardiac muscle (Fig. 1).

We investigated whether positive and negative effects of diabetes on protein mass of BCKA dehydrogenase subunits were accompanied by positive and negative effects on the expressions of genes encoding these proteins in the skeletal and cardiac muscles. Qualitative and quantitative analyses of Northern blots in the skeletal muscle (Fig. 2) showed that diabetes significantly increased the gene expression of all subunits. The increase was greater for E₂ (over 3-fold) than for either E₁α or E₁β. In sharp contrast to the skeletal muscle, the gene expression of the three subunits was not altered in the cardiac muscle of diabetic rats.

Effect of diabetes on the activity state of BCKA dehydrogenase. The above studies eliminated inverse alterations in the protein expressions of BCKA dehydrogenase as the mechanism of the different effect of diabetes on its activity. We therefore investigated the effect of diabetes on the activity state of BCKA dehydrogenase in the cardiac and skeletal muscles. The result (means ± SE in 6–8 rats) showed that diabetes increased the activity state by fourfold in the skeletal muscle (1.1 ± 0.1% vs. 4.6 ± 0.8%, P < 0.01) while decreasing the activity state by twofold in the cardiac muscle (28.2 ± 2.7% vs. 13.6 ± 1.0%, P < 0.01).

Alterations in the rate of inactivation of BCKA dehydrogenase have usually been found to be responsible for changes in the activity state. This leads to the following question: does diabetes have an inverse effect on the rates of inactivation of BCKA dehydrogenase? To answer this question, the following experiment was performed.

The rate of inactivation was studied by determining BCKA dehydrogenase activity as a function of time when ATP was added to the extracted enzyme complex.

![Fig. 1. Protein expression of branched-chain α-keto acid (BCKA) dehydrogenase subunits in cardiac (A) and skeletal muscle (B). Top: Western blots of subunits in control (C) and diabetic (D) rats. Molecular mass markers are shown at right. Bottom: densitometric analysis of Western blots of BCKA dehydrogenase subunits (E₂, E₁α, E₁β). Values are means ± SE in 4–6 rats and expressed as percentage of control (*P < 0.01 vs. control group).](http://ajpendo.physiology.org/10.22033:1)
The rate of inactivation was calculated as the first-order kinetic constant, $k \cdot \text{min}^{-1}$, of semilog plots of residual BCKA dehydrogenase activity vs. time (Fig. 3). The rate of inactivation was significantly decreased in the skeletal muscle (0.19 ± 0.02 vs. 0.07 ± 0.02, $P < 0.05$), whereas it was significantly increased in the cardiac muscle of the diabetic rats (0.88 ± 0.06 vs. 2.13 ± 0.26, $P < 0.01$).

Effect of diabetes on protein and gene expressions of BCKAD kinase. The inactivation of BCKA dehydrogenase is catalyzed by a specific kinase, which recently has been cloned (35). The enzyme phosphorylates two serine residues of BCKA dehydrogenase, one of which is responsible for the inactivation of the enzyme (44). To investigate whether the above alterations in the rates of inactivation were due to corresponding alterations in the amount of BCKAD kinase in the skeletal and cardiac muscles of diabetic rats, we determined the protein expression of the kinase in each tissue. Qualitative and quantitative analysis of Western blots showed that diabetes greatly decreased (over 2-fold) the protein expression of kinase in the skeletal muscle (Fig. 4) but significantly increased the protein expression of this enzyme in the cardiac muscle (Fig. 4).

Finally, we investigated whether the different effect of diabetes on protein expressions of the kinase in skeletal and cardiac muscles was accompanied by a similar effect on the expressions of the gene encoding this enzyme in the two muscle tissues. Qualitative and quantitative analyses of Northern blots showed that diabetes had no effect on the gene expression in the skeletal muscles (Fig. 5) but increased this expression in the cardiac muscles (Fig. 5).

DISCUSSION

This is the first study that was designed to compare the effect of diabetes on BCKA dehydrogenase activity in the cardiac and skeletal (gastrocnemius) muscles. The results show that, in diabetes, there are inverse
alterations of BCKA dehydrogenase activity in the cardiac and skeletal muscles, which correspond to similar alterations in the activity state of the enzyme in these muscles. The activity state was increased in the skeletal muscle by decreasing the amount of BCKAD kinase, whereas the activity state was decreased in the cardiac muscle by increasing the amount of BCKAD kinase. The mechanism of these protein alterations appeared to be translational or posttranslational (gene expression unchanged) in the skeletal muscle but pretranslational (gene expression increased) in the cardiac muscle. In a recent study (22), we found that diabetes decreased the amount of BCKAD kinase in rat liver without any effect on its gene expression. Therefore, among the tissues studied thus far, the cardiac muscle appears unique with its increases of protein and gene expressions of BCKAD kinase in response to diabetes.

In addition to a decrease in the protein mass of BCKAD kinase such as the one described above, there are other ways that BCKA dehydrogenase activity could be increased in the skeletal muscle. For example, Hagg et al. (12) showed that shortly after the beginning of leg exercise, oxidation of leucine was increased by twofold in human subjects. Van Hall et al. (39) showed that this exercise increased the activity state of BCKA dehydrogenase in leg muscles. Because \( \alpha \)-ketoisocaproate (KIC) is a potent inhibitor of BCKAD kinase (21), and its muscle concentration is increased in exercise (37), Shimomura et al. (37) proposed that KIC is responsible for the activation of muscle BCKA dehydrogenase during exercise. KIC is a transamination product of leucine, and the muscle concentration of leucine is shown to be increased during exercise (37). However, Kasperek (17) did not find an increase in muscle concentration of KIC during exercise, but he did find a decrease in muscle concentration of ATP. Because mitochondrial depletion of ATP results in activation of BCKA dehydrogenase (31), Kasperek (17) proposed that the same occurs in exercise. Despite this controversy, which probably reflects a methodological problem, it is reasonable to assume that both KIC and ATP concentrations influence BCKA dehydrogenase activity.

In addition to the kinase effects, our study showed another distinguishing feature in the response between the cardiac and skeletal muscles to diabetes. In the cardiac muscle, neither the gene expression nor the protein mass of any subunits of BCKA dehydrogenase was affected, whereas these expressions were all increased in the skeletal muscle. However, the results did not show a precise correlation between the increases in the gene and protein expressions of BCKA dehydroge-
nase subunits. For example, the increase was greatest for the mRNA encoding the E_2 subunit, whereas the greatest increase for protein mass was for the E_3β subunit. Recently, we found that diabetes increases the protein and gene expressions of all BCKA dehydrogenase subunits in rat liver (22). Therefore, among the tissues studied thus far, the cardiac muscle appears unique in having a BCKA dehydrogenase with molecular expressions unresponsive to the effect of diabetes.

BCKA dehydrogenase and BCKAD kinase are linked together as an enzyme complex in mitochondria. As we have discussed, diabetes has different effects on the protein mass of these enzymes in the skeletal muscle. This indicates that these alterations are not part of a general process affecting the mitochondria but are consequences of metabolic regulation.

In light of these findings, a relevant question is whether conditions such as high-protein diet and endurance training, which, like diabetes, increase BCKA dehydrogenase activity in the skeletal muscle (9, 23), are accompanied by increases in the protein and gene expressions of the enzyme subunits. Miller et al. (23) showed that a high-protein diet did not affect the protein expression of E_1α or E_2 subunits in the skeletal muscle. The protein expression of E_3β was not studied. Fujii et al. (9) showed that endurance training also did not affect the protein expression of the E_2 subunit in the skeletal muscle. The protein expressions of other subunits were not studied. However, Fujii et al. found that endurance training increased the protein expression of BCKAD kinase in the skeletal muscle without any effect on the abundance of mRNA encoding this enzyme. This is exactly what we found in diabetes, with the exception of increases in the protein expressions of all subunits of BCKA dehydrogenase (Fig. 2). Therefore, there are similarities, as well as dissimilarities, between the effects of diabetes and endurance training on the BCKA dehydrogenase complex in the skeletal muscle.

Among the tissues we studied (liver and skeletal and cardiac muscles), diabetes had the greatest effect on BCKA dehydrogenase activity in the skeletal muscle, a fourfold increase in the skeletal muscle vs. a 70% increase in the liver (22) and a twofold decrease in the cardiac muscle. We speculate that the metabolic signal for this dramatic increase in BCKA dehydrogenase activity in the skeletal muscle was probably the lowering of plasma insulin level because of insulin withdrawal in streptozotocin-treated animals. Our speculation is based on the following observations: 1) administration of insulin in humans or animals decreased leucine oxidation in a dose-dependent manner (1, 10), and 2) studies in the perfused rat hindquarter preparation, which has been used for studies of muscle metabolism, showed that addition of insulin decreases the rate of leucine oxidation (14). Because there has been no study of the effect of insulin on leucine oxidation either in perfused rat heart or in perfused rat liver, it is not yet possible to speculate whether the fall in plasma insulin level was also the signal for the alterations in BCKA dehydrogenase activity in these tissues. Previous studies have shown that factors regulating the activity of BCKA dehydrogenase complex in the liver may not be the same as those in the skeletal muscle, or vice versa. For example, Paul et al. (34) showed that clofibrate feeding reduces the activity of BCKAD kinase in the liver without affecting this activity in the skeletal muscle.

In addition to a decrease in plasma insulin level, other factors could be responsible for the increase in BCKA dehydrogenase activity in the skeletal muscle of diabetic rats. For example, in the skeletal muscle of diabetic rats, KIC concentration increases (15), and the mitochondrial concentration of ATP decreases (31). As discussed above, both of these factors could activate BCKA dehydrogenase. However, they could not account for the increases in the protein mass of the enzyme subunits observed in the skeletal muscle (Fig. 1).

Finally, the present results unravel a molecular mechanism of the body's defense against a serious metabolic consequence of diabetes. In uncontrolled diabetes there are substantial increases in blood and tissue concentrations of branched-chain amino acids (4, 42), which lead to increased production of BCKA (15). BCKA are neurotoxic, as evidenced in maple syrup urine disease (6). Therefore, an increase in BCKA dehydrogenase activity brought on by an increase in its protein expression together with a decrease in the protein expression of its associated kinase in the skeletal muscle could be considered a protective mechanism. In view of the general belief that the skeletal muscle is the major site for oxidation of branched-chain amino acids (38), a severalfold increase in its BCKA dehydrogenase activity is likely to play a major role in the increase of whole body oxidation of BCKA observed in patients with diabetes mellitus (16, 20, 24). However, our previous studies (29, 40, 41) have shown that, whenever there is an increase in BCKA dehydrogenase activity, there is also an increase in protein catabolism. Therefore, the increased dehydrogenase activity in uncontrolled diabetes is most likely an early sign of protein wasting in the skeletal muscle. Protein wasting would have become apparent if we had allowed a longer period of uncontrolled diabetes. In the same context, an increase in the protein expression of BCKAD kinase in the cardiac muscle may be a sign of protein sparing in this tissue during uncontrolled diabetes.

We thank Dr. Robert H. Lane (University of Pittsburgh School of Medicine) for the design and supply of the kinase primers. This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-15855. Present address of Y. B. Lombardo: School of Biochemistry, Ciudad Universitaria, 3000 Santa Fe, Argentina; present address of H. S. Paul: Biomed Research Technologies, Wexford, PA 15090. Address for correspondence and reprint requests: S. A. Adibi, UPMC Health System, 200 Lothrop St., MUH E-321, Pittsburgh, PA 15213 (E-mail: adibi@med1-dept-med.pitt.edu). Received 16 February 1999; accepted in final form 2 June 1999.

REFERENCES


