Vascular endothelium-derived factors and arterial stiffness in strength- and endurance-trained men

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Otsuki T, Maeda S, Iemitsu M, Saito Y, Tanimura Y, Ajisaka R, Miyauchi T. Vascular endothelium-derived factors and arterial stiffness in strength- and endurance-trained men. Am J Physiol Heart Circ Physiol 292: H786–H791, 2007. First published September 22, 2006; doi:10.1152/ajpheart.00678.2006—Arterial stiffness is higher in strength-trained humans and lower in endurance-trained humans. However, the mechanisms underlying these different adaptations are unclear. Vascular endothelium-derived factors, such as endothelin-1 (ET-1) and nitric oxide (NO), play an important role in the regulation of vascular tonus. We hypothesized that endogenous ET-1 and NO participate in the adaptation of arterial stiffness in different types of exercise training. The purpose of this study was to investigate plasma ET-1 and NO concentrations and arterial stiffness in strength- and endurance-trained men. Young strength-trained athletes (SA; n = 11), endurance-trained athletes (EA; n = 12), and sedentary control men (C; n = 12) participated in this study. Maximal handgrip strength in SA and maximal oxygen uptake in EA were markedly greater than in C. Aortic pulse-wave velocity, which is an established index of arterial stiffness, was higher in SA and lower in EA than in C. Additionally, we measured systemic arterial compliance (SAC) using carotid arteryplanationometry and Doppler echocardiography, because arterial stiffness is a primary determinant of the compliance. SAC was lower in SA and higher in EA compared with that in C. Plasma ET-1 concentrations were higher in SA compared with C and EA. We did not find significant differences in plasma NO concentrations (measured as the stable end product of NO, i.e., nitrite/nitrate). The relationships of plasma ET-1 concentrations to aortic pulse-wave velocity and SAC were linear. These results suggest that differences in endogenous ET-1 may partly participate in the mechanism underlying different adaptations of arterial stiffness in strength- and endurance-trained men.

arterial compliance; endothelin-1; nitric oxide; pulse-wave velocity

Vascular endothelial cells play an important role in the regulation of vascular activity by producing vasoactive substances, such as endothelin-1 (ET-1) and nitric oxide (NO). ET-1 is a potent vasoconstrictor peptide produced by vascular endothelial cells (22). It has been reported that systemic administration of an endothelin receptor antagonist significantly decreased systemic blood pressure and peripheral vascular resistance in healthy humans, strongly suggesting that endogenously generated ET-1 contributes to basal vascular tonus in humans (7). Previous study has demonstrated that plasma ET-1 concentrations increase in some human cardiovascular diseases (4). We have reported that plasma ET-1 concentrations increase with aging even in normal subjects (18, 23). On the other hand, endurance exercise training decreases plasma ET-1 concentrations (16, 18). NO produced by vascular endothelial cells has a potent vasodilator effect and plays an important role in the regulation of platelet-vessel wall interactions and vascular resistance and growth (24). The plasma NO [measured as the stable end product of NO, i.e., nitrite/nitrate (NOx)] concentrations decrease in some human cardiovascular diseases, e.g., essential hypertension (25), and NO bioavailability may decrease with aging (31). We have previously demonstrated that plasma NO (NOx) concentrations are increased by endurance training in healthy humans (16, 19). It has also been reported that endurance training is associated with improved NO bioavailability in healthy humans (8, 10). Therefore, altered plasma ET-1 and NO concentrations may have important clinical significance for both the physiological and pathological states.

Since ET-1 and NO are implicated in the regulation of vascular tonus, it is reasonable to hypothesize that ET-1 and NO participate in the mechanisms underlying the different adaptations of arterial stiffness to strength and endurance training. However, the relationships of the plasma concentrations of these endothelium-derived factors to the adaptations of arterial stiffness by different types of exercise training remain unclear. The primary aim of this study was to investigate plasma ET-1 and NO concentrations and arterial stiffness in strength- and endurance-trained men. To test our hypothesis, we measured plasma ET-1 and NOx concentrations; aortic pulse-wave velocity (PWV), a traditional index of arterial stiffness; and systemic arterial compliance (SAC) in strength-trained and endurance-trained men.

METHODS

Subjects. Eleven male shot put, hammer, or javelin throwers (strength-trained athletes; SA), twelve male long or middle distance
runners (endurance-trained athletes; EA), and twelve sedentary (untrained) control men (C) volunteered to participate in this study. All of the athletes were intercollegiate athletes belonging to track and field teams, and their competitive sports careers were longer than 2 years. The training volume and intensity in the SA group were 5.1 ± 0.1 sessions/wk (3.1 h/session) at an average intensity of 15 on the rating of perceived exertion scale (i.e., hard). Those in the EA group were 5.5 ± 0.3 sessions/wk (2.4 ± 0.3 h/session) and the rating of 15–17 (i.e., hard–very hard). Athletic training in the SA group consisted of throwing, sprint, and plyometric training and skill practice. The SA group had been performing vigorous whole-body weight training (3 sessions/wk) in addition to their athletic training. The training in the EA group mainly consisted of some kind of running training, such as long-distance running and interval training. The athletes who had been concurrently and regularly performing both types of training (i.e., cross-training) were excluded. The C group had a sedentary lifestyle (no regular physical activity) for at least 2 years. All subjects were free of signs, symptoms, and history of any overt chronic diseases. None of the participants had a history of smoking and none were currently taking any medications, anabolic steroids, or other performance-enhancing drugs. Before all measurements, subjects refrained from alcohol consumption and intense physical activity (exercise) for 24 h and caffeine consumption for 4 h to avoid immediate (acute) effects.

This study was approved by the Ethics Committee of the Institute of Health and Sport Sciences of the University of Tsukuba. This study conformed to the principles outlined in the Helsinki Declaration. All subjects provided written, informed consent before inclusion in this study.

Blood biochemistry. All participants were instructed to stop oral intake, without water, overnight 12 h before blood sampling. Each blood sample was placed in a chilled tube containing aprotinin (300 kallikrein-inactivating U/ml) and EDTA (2 mg/ml) and was then centrifuged at 2,000 × g for 15 min at 4°C. The plasma was stored at −80°C until assay. Plasma concentrations of ET-1 were determined by using a sandwich-EIA Kit (Immuno-Biological Laboratories, Fujioka, Japan). The ET-1 assay was carried out as previously described by our laboratory (17). The intra-assay coefficients of variation of the ET-1 assay were 11% in our laboratory (18). Plasma concentrations of NOx were determined by using a NO (NO2⁻/NO₃⁻) assay kit (R&D Systems, Minneapolis, MN). The NOx assay was carried out according to the manufacturer’s instructions. Serum concentrations of cholesterol, triglycerides, and insulin and plasma concentrations of glucose were determined by using the standard enzymatic techniques.

Aortic PWV. Aortic PWV was measured at constant room temperature (25°C) using application tonometry as previously described (5) with minor modifications. Briefly, carotid and femoral arterial pulse waves were obtained in triplicate using arterial application tonometry incorporating an array of 15 transducers (formPWV/ABI; Colin Medical Technology, Komaki, Japan) after a resting period of at least 20 min. The distance traveled by the pulse waves was assessed in triplicate with a random zero-length measurement over the surface of the body with a nonelastic tape measure. Pulse-wave transit time was determined from the time delay between the proximal and distal “foot” waveforms. The foot of the wave was identified as the commencement of the sharp systolic upstroke, which was automatically detected. Aortic PWV was calculated as the distance divided by the transit time.

SAC. SAC was measured by using carotid artery application tonometry and Doppler echocardiography as previously described with minor modifications (3, 14). Briefly, carotid artery pressure waveforms were obtained by using application tonometry (formPWV/ABI; Colin Medical Technology) after a resting period of at least 20 min. At the time of waveforms recording, brachial arterial systolic, diastolic, and mean blood pressure (SBP, DBP, and MBP, respectively) were measured by using oscillometry (formPWV/ABI; Colin Medical Technology). The pressure signal obtained by tonometry was calibrated by equating the carotid MBP and DBP to brachial artery values. SAC was calculated as follows: SAC = Ad/dP × R, where Ad is the area under an arbitrary portion of the diastolic pressure waveform, dP is the pressure change in this portion, and R is systemic vascular resistance given as MBP divided by mean blood flow. Since the calculation of SAC is based on the assumption that the diastolic pressure decay is a monoexponential function of time, the diastolic pressure waveform was used to calculate SAC according to the previous studies (3, 14). Mean blood flow was measured by using a Doppler echocardiographic system (EnVisor; Koninklijke Philips Electronics, Eindhoven, Netherlands) as previously described by our laboratory (26). The insertion point of the aortic valve tips at end diastole was defined by two-dimensional imaging in the parasternal long-axis view with a 3.5-MHz transducer, and the M-mode echocardiogram at that level was stored into the computer. Doppler ultrasonographic flow velocity curves in the ascending aorta were simultaneously recorded by using a 1.9-MHz probe held in the suprasternal notch. Mean blood flow was calculated as a product of the aortic cross-sectional area and the mean flow velocity (ImageJ; National Institutes of Health, Bethesda, MD).

Maximal handgrip strength and maximal oxygen uptake. Maximal handgrip strength and maximal oxygen uptake were measured after blood sampling, and PWV and SAC measurements were taken. Maximal handgrip strength was determined by using a hand dynamometer (HK51020; SUNCREA, Tokyo, Japan). Two maximal contractions, each lasting 3 to 5 s and at least 15 s apart, were performed by each hand. The maximal strength score achieved from the two trials was taken as the maximal handgrip strength.

Maximal oxygen uptake was determined by using incremental cycling to exhaustion (a 3 min at 80 W, with a 30-W increase every 3 min) by monitoring breath-by-breath oxygen consumption and carbon dioxide production (AE280S; Minato Medical Science, Osaka, Japan), heart rate, and ratings of perceived exertion (Borg scale).

Statistical analysis. Data are expressed as means ± SE. Statistical analysis was carried out using one-way ANOVA followed by Fisher protected least significant differences test for multiple comparisons. The blood pressure-independent effects of training on PWV and SAC were tested by using analysis of covariance (ANCOVA). Stepwise regression analyses were used to determine significant, independent physiological correlates for each of the arterial stiffness measurements. The partial correlation analysis was used to determine whether the associations of plasma levels of vascular endothelium-derived factors with PWV and SAC were independent of blood pressure. P < 0.05 was accepted as significant.

RESULTS

Table 1 summarizes the characteristics of all groups. Body weight, body mass index (BMI), and body circumferences were greater in SA compared with C and EA, although there were no significant differences in age and height. Serum HDL cholesterol concentrations were higher in EA than in SA. Maximal handgrip strength was greater in SA than in C and EA. Maximal oxygen uptake was higher in EA compared with C and SA. Table 2 shows the hemodynamics of all groups. Blood pressure was higher in SA compared with C and EA. These results indicate that SA and EA were morphologically and functionally adapted to their respective exercise training. ANOVA showed that strength- and endurance-exercise training affected aortic PWV, as an index of arterial stiffness (Fig. 1A; F = 19.9, and P < 0.05). The increased MBP and DBP enhance arterial stiffness. Indeed, we found that aortic PWV was related to MBP (r = 0.44, and P < 0.05) and DBP (r = 0.46, and P < 0.05). ANCOVA, however, revealed that the effect of exercise training on aortic PWV was statistically
Table 1. Characteristics of strength- and endurance-trained men and sedentary control men

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Sedentary</th>
<th>Endurance</th>
<th>Strength</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>21.3±0.7</td>
<td>20.0±0.2</td>
<td>21.0±0.5</td>
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<tr>
<td>Height, cm</td>
<td>172±2</td>
<td>171.2</td>
<td>177±1</td>
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<tr>
<td>Weight, kg</td>
<td>65.7±2.0</td>
<td>61.8±1.5</td>
<td>87.3±1.5†</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>22.1±0.5</td>
<td>21.1±0.3</td>
<td>27.9±0.6†</td>
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<tr>
<td>Chest, cm</td>
<td>87±1</td>
<td>85±1</td>
<td>103±2†</td>
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<tr>
<td>Waist, cm</td>
<td>75±1</td>
<td>71±1</td>
<td>90±3†</td>
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<td>Hip, cm</td>
<td>95±1</td>
<td>91±1*</td>
<td>106±2†</td>
</tr>
<tr>
<td>Upper arm, cm</td>
<td>26.8±0.4</td>
<td>25.5±0.5</td>
<td>35.0±1.3†</td>
</tr>
<tr>
<td>Thigh, cm</td>
<td>50.7±0.7</td>
<td>50.3±0.6</td>
<td>58.9±1.0†</td>
</tr>
<tr>
<td>Total cholesterol, mg/dl</td>
<td>178±9</td>
<td>181±8</td>
<td>170±9</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dl</td>
<td>61±4</td>
<td>70±4</td>
<td>51±3‡</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dl</td>
<td>103±11</td>
<td>98±6</td>
<td>102±9</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>90±3</td>
<td>90±2</td>
<td>89±3</td>
</tr>
<tr>
<td>Insulin, μU/ml</td>
<td>11.7±1.2</td>
<td>9.0±1.3</td>
<td>9.2±1.4</td>
</tr>
<tr>
<td>Maximal oxygen uptake, ml·kg⁻¹·min⁻¹</td>
<td>45.2±1.6</td>
<td>59.4±1.0†</td>
<td>43.0±1.0†</td>
</tr>
<tr>
<td>Maximal hand-grip strength, kg</td>
<td>42.6±2.1</td>
<td>42.1±1.5</td>
<td>58.0±1.7†</td>
</tr>
</tbody>
</table>

Values are means ± SE. Sedentary, sedentary control men; Endurance, endurance-trained men; Strength, Strength-trained men. *P < 0.05 vs. Sedentary; †P < 0.05 vs. Endurance.

independent of MBP (F = 15.5, and P < 0.05) and DBP (F = 13.8, and P < 0.05). To confirm these relationships, we measured their SAC, because arterial stiffness is a primary determinant of the compliance. The variability of SAC was also shown to be associated with exercise training by using ANOVA (Fig. 1; F = 9.0, and P < 0.05). SAC correlated with MBP (r = −0.36, and P < 0.05) but was not statistically associated with DBP (r = −0.28, and P = 0.103). The effect of exercise training on SAC, which was tested by using ANCOVA, was also independent of MBP and DBP (F = 6.7, and P < 0.05; and F = 6.8, and P < 0.05, respectively). In multiple comparisons, aortic PWV was higher in SA than in C and EA; PWV was also lower in EA compared with that in C. SAC was lower in SA and higher in EA compared with that in C. Figure 2 shows plasma ET-1 levels in all groups. Plasma ET-1 concentrations were higher in SA compared with C and EA. We did not find the significant relation of plasma ET-1 concentrations to MBP (r = 0.29, and P = 0.086) and DBP (r = 0.26, and P = 0.140). The relationships of plasma ET-1 concentrations to aortic PWV and SAC were linear (Fig. 3). Additionally, partial correlation analysis revealed that plasma ET-1 concentrations were related to aortic PWV, even after adjusting for MBP (r = 0.38, and P < 0.05) and DBP (r = 0.46, and P < 0.05), respectively. We also found that the association of plasma ET-1 concentrations with SAC was statistically independent of MBP (r = −0.34, and P < 0.05) and DBP (r = −0.41, and P < 0.05). We did not find a

Table 2. Hemodynamics of strength- and endurance-trained men and sedentary control men

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Sedentary</th>
<th>Endurance</th>
<th>Strength</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>119±3</td>
<td>115±3</td>
<td>128±4†</td>
</tr>
<tr>
<td>Mean blood pressure, mmHg</td>
<td>86±2</td>
<td>84±2</td>
<td>90±2</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg</td>
<td>66±2</td>
<td>61±2</td>
<td>68±2†</td>
</tr>
<tr>
<td>Pulse pressure, mmHg</td>
<td>53±1</td>
<td>54±1</td>
<td>60±2†</td>
</tr>
<tr>
<td>Heart rate, beat/min</td>
<td>65±4</td>
<td>59±3</td>
<td>60±2</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.05 vs. Sedentary; †P < 0.05 vs. Endurance.
significant effect of training on plasma NOx concentrations (Fig. 4).

To establish which factors were independent predictors of arterial stiffness in overall population, we performed stepwise regression analyses of age, BMI, blood pressure, and heart rate; serum concentrations of cholesterol, triglycerides, and insulin; and plasma concentrations of glucose, ET-1, and NOx to arterial stiffness. Coefficients of determination ($r^2$) were 0.61 (aortic PWV) and 0.59 (SAC), respectively. The Variables that were entered for aortic PWV were plasma ET-1 concentrations, age, SBP, and BMI. Plasma concentrations of ET-1 and serum concentrations of HDL and LDL cholesterol and insulin were entered for SAC.

DISCUSSION

It is now well established that increased vascular tonus and proliferation of vascular smooth muscle cells increase arterial stiffness and consequently decrease arterial compliance (28, 33). ET-1 has potent vasoconstrictor activity and potent proliferative effects on vascular smooth muscle cells (22). Furthermore, it has been reported that arterial stiffness was increased by intra-arterial infusion of ET-1 and decreased by the administration of ET-1 receptor antagonist (20, 34). These findings suggest that endogenous ET-1 participates in the regulation of arterial stiffness. In this study, we found that aortic PWV was higher and SAC was lower in strength-trained men and that aortic PWV was lower and SAC was higher in endurance-trained men. We have also demonstrated that plasma concentrations of ET-1 were significantly higher in strength-trained men than in endurance-trained or sedentary men. The relationships of aortic PWV and SAC to plasma ET-1 concentrations were linear. Furthermore, stepwise regression analysis and partial regression analysis revealed that the associations of plasma ET-1 concentrations with aortic PWV and SAC were independent of blood pressure. These results suggest that differences in plasma ET-1 levels may be involved in the mechanism underlying adaptations of arterial stiffness to different types of exercise training.

Previous studies have reported the effects of ET-1 infusion, endothelin receptor blockade, and inhibition of NO production on arterial stiffness (11, 20, 30, 34, 35). Recently, we have demonstrated that the inhibition of NO production appeared to have no effect on the acute decrease in middle-sized arterial stiffness with low-intensity, short-duration exercise, although its decrease was induced mainly by regional factors (30). Wilkinson et al. (35) have reported that PWV increased by only 3% during the intra-arterial infusion of NO synthase inhibitor (10 μM/min). In the same experimental setting, they have also demonstrated that the administration of ET-1 (10 pM/min) increased PWV by 12% and that treatment with endothelin A receptor antagonist (40 nM/min) reduced PWV by 12% (20). These findings suggest that ET-1 may be a more important factor to the regulation of arterial stiffness than NO (20). In the present study, we showed the increased arterial stiffness and plasma ET-1 concentrations in strength-trained men. On the basis of the results from past studies plus the present results, we propose that the higher ET-1 production in strength-trained men increases arterial stiffness.

The mechanism underlying the increased plasma ET-1 concentrations in strength-trained men remains unclear. It has been reported that arterial blood pressure increases to 340/240 mmHg during bouts of high-intensity strength exercise (15). After such high-intensity strength exercise, arterial stiffness increases acutely for at least 30 min, although arterial blood pressure rapidly returns to the preexercise basal level (6). Since ET-1 has potent vasoconstrictor and pressor activities (22), it is possible that endogenous

![Fig. 3. Relationships of plasma endothelin-1 concentrations to aortic pulse-wave velocity (A; $n=35$; $r=0.46$; $y=0.62x+4.9$; $P<0.05$) and systemic arterial compliance (B; $n=35$; $r=-0.41$; $y=-0.38x+1.9$; $P<0.05$) were linear. The C (○), EA (●), and SA (●) groups are shown.](http://ajpheart.physiology.org/)
ET-1 participates in increased blood pressure and arterial stiffness during and following high-intensity strength exercise. The intermittent repetition of high-intensity strength exercises (i.e., strength training) might cause an elevation of basal plasma ET-1 levels in strength-trained men.

The conclusions drawn in this study come from examination of plasma ET-1 concentrations. It is generally accepted that ET-1 acts predominantly in autocrine and paracrine manners, and the secretion of ET-1 by endothelial cells is polarized toward the underlying vascular smooth muscle (22). Consequently, plasma levels are largely the result of spillover from the vascular endothelium into the bloodstream. Since only ~20% of generated ET-1 is secreted intraluminally (36), plasma ET-1 concentrations are very low. However, the plasma levels of ET-1 are associated with disease severity (4) and increase with aging even in normal subjects (18, 23). Therefore, the alteration of plasma ET-1 concentrations may have important clinical significance in physiology and/or pathophysiology. Taken together, these data suggest that the differences in plasma ET-1 concentration may be involved in the different adaptations of arterial stiffness to strength training and endurance training.

Increased arterial stiffness implicates in the pathophysiology of cardiovascular disease (2, 12). In this study, arterial stiffness was higher and arterial compliance was lower in strength-trained men compared with endurance-trained men and sedentary control men. Additionally, blood pressure was also higher in the strength-trained men. It is possible that the increased arterial stiffness is one of the physiological adaptations to the intense increase in blood pressure during strengthening exercise. However, at resting conditions, it is unlikely that the increased arterial stiffness and blood pressure are beneficial to vessels and heart. The Bogalusa Heart Study has reported that intima-media thickness of carotid arteries in healthy young adults was associated with the cumulative burden of cardiovascular risk factors since childhood (13). The Young Finns Study has identified an association between school-age risk variables and intima-media thickness of carotid arteries at 36 yr, even after adjustment for contemporaneous risk variables (27). Recently, Miyachi et al. (21) have reported that the decreased arterial compliance caused by strength training dissipated to the basal level (the level before strength training) 2 mo after cessation of strength training in young healthy men. Furthermore, we have reported that the decreased plasma ET-1 levels observed in endurance training returned to the basal level 2 mo after cessation of training (16). Therefore, we believe that increased arterial stiffness and plasma ET-1 concentrations in strength-trained men are physiological adaptations to their competitive sports.

The present investigation has the following study limitations. First, blood pressure in strength-trained men was higher than in endurance-trained men and sedentary peers. This might influence the main results in this study. However, in this study, we performed statistical analysis in consideration of the differences in blood pressure. ANCOVA revealed that the effects of exercise training on arterial stiffness were statistically independent of blood pressure. Additionally, stepwise regression analysis was used to determine the blood pressure-independent association between plasma ET-1 concentrations and arterial stiffness. Although the sample size in the present study may not be enough to use this method, the results of partial correlation analysis did not conflict with that of stepwise regression analysis. The conceivable next steps would be to compare arterial stiffness and plasma ET-1 concentrations between blood pressure-matched groups to determine the independent role of ET-1 on arterial stiffness. Second, it is difficult to identify which arteries determine the value of SAC. Stergiopulos et al. (29) has reported that the aortic occlusion at the aortic trifurcation did not change SAC but that the occlusion at the level of proximal descending aorta induced a 40% reduction of SAC. Thus it is considered that compliance of the descending aorta at least participates in SAC and that ascending aorta and/or the ramified arteries such as carotid artery would also be implicated in SAC.

In conclusion, the present study has demonstrated that plasma concentrations of ET-1 were significantly higher in strength-trained men than in endurance-trained or sedentary men. We have also demonstrated that arterial stiffness was increased in strength-trained men and reduced in endurance-trained men. The association of arterial stiffness with plasma ET-1 concentrations was linear and statistically independent of blood pressure. We propose that differences in plasma concentrations of ET-1 may partly participate in the mechanism underlying different adaptations of arterial stiffness to strength and endurance training.

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REFERENCES