Baroreceptor afferent discharge in the pregnant rat

TINA HINES
University of Pittsburgh School of Nursing, Pittsburgh, Pennsylvania 15261

Hines, Tina. Baroreceptor afferent discharge in the pregnant rat. Am J Physiol Regulatory Integrative Comp Physiol 278: R1433–R1440, 2000.—Pregnancy is associated with marked alterations in cardiovascular function, including a large increase in plasma volume and cardiac output but a decrease in arterial blood pressure. These changes are measured early in gestation and are maintained until late pregnancy. The maintenance of these hemodynamic changes suggests that autonomic regulatory mechanisms are also altered during gestation. It is known that the arterial baroreflex rapidly resets when arterial pressure is lowered (29); thus, it should be expected that the reduction in prevailing blood pressure during pregnancy would shift the baroreflex curve to a lower pressure set point. Baroreflex resetting may thus allow afferent discharge in pregnant rats, and this involves a more rapid adaptation to a pressure stimulus.

baroreflex; aortic depressor nerve; phenylephrine; nitroprusside

PREGNANCY IS ASSOCIATED with marked alterations in cardiovascular function, including a large increase in plasma volume and cardiac output but a decrease in arterial blood pressure. These changes are measured early in gestation and are maintained until late pregnancy. The maintenance of these hemodynamic changes suggests that autonomic regulatory mechanisms are also altered during gestation. It is known that the arterial baroreflex rapidly resets when arterial pressure is lowered (29); thus, it should be expected that the reduction in prevailing blood pressure during pregnancy would shift the baroreflex curve to a lower pressure set point. Baroreflex resetting may thus allow afferent discharge in pregnant rats, and this involves a more rapid adaptation to a pressure stimulus.

baroreflex; aortic depressor nerve; phenylephrine; nitroprusside

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
response to a given pressure stimulus is attenuated in pregnant compared with virgin rats.

METHODS

Virgin Sprague-Dawley (Hilltop Animal Labs, Scottsdale, PA) rats were mated (n = 9) or served as age-matched controls (n = 9). Day 1 of pregnancy was determined by the presence of sperm in the vaginal smear and experiments were conducted on gestational day 20 (rat pregnancy = 22 days). Animals were maintained on a 12:12-h light-dark cycle and were fed standard rat chow during the gestational period.

Surgical procedures. Rats were anesthetized with pentobarbital sodium (35 mg/kg, ip), and supplemental anesthesia (10 mg/kg iv) was administered as necessary to maintain a stable blood pressure and absence of reflex withdrawal to hindpaw pinch. Catheters were inserted into a femoral artery and both femoral veins for measurement of arterial pressure and drug infusions, respectively. The trachea was cannulated, and animals breathed room air unassisted in all experiments. Through a midline cervical incision, the left aortic depressor nerve (ADN) was gently dissected caudally from its junction with the superior laryngeal nerve. The ADN was mounted intact on a bipolar stainless steel electrode, and multifiber nerve activity was amplified 5,000–10,000 times, filtered between 100 and 1,000 Hz, and fed to an oscilloscope, audio monitor, and signal rectifier. After an optimum signal was obtained, the nerve was protected with lightweight silicon impression material (Coltene/Whaledent, New York, NY). Rectified aortic depressor nerve activity (ADNA) was sampled at 8 kHz and along with arterial pressure was digitized and displayed on a computer monitor (Spike2; Cambridge Electronic Design, Cambridge, UK).

Experimental procedures. Arterial pressure and ADNA were recorded in response to constant cumulative infusions of phenylephrine (PE, 1.5–24 µg·kg$^{-1}·$min$^{-1}$) and sodium nitroprusside (SNP, 5–80 µg·kg$^{-1}·$min$^{-1}$). Each dose was infused for 10 min at an initial rate of 1.7 µl/min. Doses were increased by successively doubling the infusion rate so that the total volume infused was 0.53 ml. Arterial pressure and ADNA were measured during the first and last minutes of each dose infusion. To assess the baroreceptor response to an acute increase in arterial pressure, PE (1–16 µg/kg) and SNP (0.5–16 µg/kg) were also administered as intravenous bolus injections. Doses of PE or SNP were injected in random order and separated by 10 min. Postmortem nerve activity was measured after euthanasia (KCl) and was subtracted from all raw ADNA levels before analysis.

Data analysis. HR was derived from the arterial pressure pulse and displayed as a marker on the data file. During constant infusions, MAP, HR, and rectified ADNA were calculated (1-s bins) during a 60-s baseline recording and during the first and last minutes of each dose infusion. Baseline MAP, HR, and ADNA during bolus dose administration were averaged in the 30 s preceding an injection, and changes in these variables were measured at the peak of their response (=5- to 10-s segments). Statistical analysis of baseline values was done using analysis of variance (ANOVA). Changes in variables in response to PE and SNP infusions and bolus doses were compared using repeated-measures ANOVA. Slopes and y-intercepts relating MAP to HR or ADNA were calculated using least squares linear regression analysis and compared between groups by analysis of covariance (Statistica; StatSoft, Tulsa, OK). To assess the between-group differences in adaptation of baroreceptor discharge, two different analyses were used. Changes in ADNA (expressed as percent of baseline) from the 1st to the 10th minute of each dose infusion were compared between groups by ANOVA. In addition, the ADNA dose response measured during the 1st minute of each drug dose was compared with the dose response measured during the 10th minute by repeated-measures ANOVA. A nonlinear logistic analysis of the ADNA/MAP correlation during the 10th minute of each dose was attempted; however, maximum ADNA values were not achieved in virgin rats at the doses of PE used; therefore, a complete statistical comparison of logistic parameters between groups was not possible. Nevertheless, we did attempt to estimate certain logistical parameters by artificially setting maxima in virgin rats using statistical extrapolation of missing data points (Statistica). Data are presented as means ± SE, and significance was set at P < 0.05.

All experimental procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, as approved by the Council of the American Physiological Society, and were approved by the Animal Care and Use Committee of the University of Pittsburgh.

RESULTS

Effects on constant infusions of PE and SNP on ADNA. Resting MAP was significantly lower in pregnant compared with virgin rats before constant infusions (Fig. 1). Although MAP remained lower in pregnant rats, constant, cumulative infusions of PE and SNP (10 min/dose) evoked dose-dependent increases and decreases in MAP that were similar in both groups (Fig. 1).

ADNA (expressed as a percent of basal levels) also increased and decreased in a dose-dependent manner in response to PE and SNP infusions (Fig. 2). In pregnant compared with virgin rats, baroreceptors began to demonstrate adaptation to the pressure stimulus at lower doses of both agonists. The inset in Fig. 2 compares the change in ADNA from the 1st to the 10th minute of each dose in pregnant and virgin rats. Changes in ADNA over the course of the 10-min period were analyzed separately for the two drugs and were compared for each group.
The more rapid adaptation to the pressure stimulus in pregnant rats resulted in significant between-groups differences in the ADNA dose-response curve measured during the 10th minute of each dose infusion (ANOVA, P < 0.05). Some adaptation was also seen in baroreceptors from virgin animals at the highest dose of PE and SNP (Fig. 2). Comparison of the ADNA dose-response relationship during the 1st minute of each dose was not significantly different between groups (P < 0.05). The more rapid adaptation to the pressure stimulus in pregnant rats resulted in significant between-groups differences in the ADNA dose-response curve measured during the 10th minute of each dose infusion (ANOVA, P < 0.05). Some adaptation was also seen in baroreceptors from virgin animals at the highest dose of PE and SNP (Fig. 2). Comparison of the ADNA dose-response relationship during the 1st minute of each dose was not significantly different between groups (P < 0.05). The more rapid adaptation to the pressure stimulus in pregnant rats resulted in significant between-groups differences in the ADNA dose-response curve measured during the 10th minute of each dose infusion (ANOVA, P < 0.05). Some adaptation was also seen in baroreceptors from virgin animals at the highest dose of PE and SNP (Fig. 2). Comparison of the ADNA dose-response relationship during the 1st minute of each dose was not significantly different between groups (P < 0.05). The more rapid adaptation to the pressure stimulus in pregnant rats resulted in significant between-groups differences in the ADNA dose-response curve measured during the 10th minute of each dose infusion (ANOVA, P < 0.05). Some adaptation was also seen in baroreceptors from virgin animals at the highest dose of PE and SNP (Fig. 2). Comparison of the ADNA dose-response relationship during the 1st minute of each dose was not significantly different between groups (P < 0.05). The more rapid adaptation to the pressure stimulus in pregnant rats resulted in significant between-groups differences in the ADNA dose-response curve measured during the 10th minute of each dose infusion (ANOVA, P < 0.05). Some adaptation was also seen in baroreceptors from virgin animals at the highest dose of PE and SNP (Fig. 2). Comparison of the ADNA dose-response relationship during the 1st minute of each dose was not significantly different between groups (P < 0.05). The more rapid adaptation to the pressure stimulus in pregnant rats resulted in significant between-groups differences in the ADNA dose-response curve measured during the 10th minute of each dose infusion (ANOVA, P < 0.05). Some adaptation was also seen in baroreceptors from virgin animals at the highest dose of PE and SNP (Fig. 2). Comparison of the ADNA dose-response relationship during the 1st minute of each dose was not significantly different between groups (P < 0.05). The more rapid adaptation to the pressure stimulus in pregnant rats resulted in significant between-groups differences in the ADNA dose-response curve measured during the 10th minute of each dose infusion (ANOVA, P < 0.05). Some adaptation was also seen in baroreceptors from virgin animals at the highest dose of PE and SNP (Fig. 2). Comparison of the ADNA dose-response relationship during the 1st minute of each dose was not significantly different between groups (P < 0.05). The more rapid adaptation to the pressure stimulus in pregnant rats resulted in significant between-groups differences in the ADNA dose-response curve measured during the 10th minute of each dose infusion (ANOVA, P < 0.05). Some adaptation was also seen in baroreceptors from virgin animals at the highest dose of PE and SNP (Fig. 2). Comparison of the ADNA dose-response relationship during the 1st minute of each dose was not significant between groups by repeated-measures ANOVA.

Because ADNA in virgin rats did not reach a maximum at the doses employed, statistical comparison of nonlinear logistic parameters was not possible. To estimate other logistic parameters, however, maximum values in virgin rats were artificially set using statistical extrapolation for missing data points from the measurements made during the 10th minute of each dose (Statistica), and logistic curves were derived (Delta Graph; SPSS, San Raphel, CA). These parameters will have to be confirmed in future studies but do suggest a reduction in the slope (pregnant = 2.99 ± 1.3 vs. virgin = 6.13 ± 2.5 ADNA as % of baseline/mmHg) and higher minimum ADNA (pregnant = 60.7 ± 12.8%; virgin = 49.2 ± 10.0%) in pregnant compared with virgin rats.

The correlation between ADNA and MAP was analyzed by linear regression using data points obtained during the 10th minute of each dose infusion of PE and SNP (Fig. 4). In response to comparable increases in MAP (Fig. 4A), ADNA levels were higher in pregnant compared with virgin rats, but the slope of the relationship was significantly blunted in the gravid rats (pregnant = 0.67 ± 0.13, virgin = 1.69 ± 0.26; P < 0.05). Although the magnitude of the pressor response evoked by PE infusion was less in pregnant rats, the blunted slope predicted that at higher pressures, ADNA would
be reduced in pregnant compared with virgin animals. The slopes of the ADNA/MAP relationships during SNP infusion (Fig. 4B) were similar in the two groups (pregnant = 0.59 ± 0.12, virgin = 0.92 ± 0.23; P = 0.08) but shifted to the left in pregnant rats (y-intercept: pregnant = 31.6 ± 10.2, virgin = 1.09 ± 12.6; P < 0.05).

Because absolute levels of MAP were lower in pregnant rats during PE and SNP infusions, ADNA was also plotted as a function of percent change in MAP in both groups (Fig. 5). Analyzed in this fashion, the slopes of the ADNA/MAP relationship were significantly attenuated in pregnant compared with virgin rats during PE infusion (Fig. 5A: pregnant slope = 0.78 ± 0.2, virgin = 1.88 ± 0.33; P < 0.05) and SNP infusion (Fig. 5B: pregnant slope = 0.75 ± 0.13, virgin = 1.17 ± 0.19; P < 0.05).

Baseline HR before constant infusions was not different in the two groups (Fig. 6). The slope of the correlation between HR and MAP was not different from zero in the pregnant group in response to increases in arterial pressure with PE and differed significantly from the slope in virgin rats (Fig. 6A: pregnant slope = −0.09 ± 0.06, virgin slope = −0.54 ± 0.2; P < 0.05). During SNP infusions, there was little change in HR in either group, and slopes were not different (Fig. 6B: pregnant slope = −0.02 ± 0.02, virgin slope = −0.38 ± 0.18).

Effects of bolus injections of PE and SNP on ADNA. Basal MAP before bolus injection of PE and SNP was significantly lower in pregnant (105.9 ± 4.2 mmHg) compared with virgin rats (123.5 ± 2.9 mmHg). Changes in MAP and ADNA during bolus injections of both PE (Fig. 7A) and SNP injections (Fig. 7B) were similar in both groups. The slopes of the correlations between ADNA and MAP, plotted as a function of percent change, during bolus PE (Fig. 7A: pregnant = 2.75 ± 0.98, virgin = 4.40 ± 1.5) and SNP injections (Fig. 7B: pregnant = 2.0 ± 0.92, virgin = 1.54 ± 1.1) were also not different between groups. There was a tendency toward an attenuated slope in pregnant rats during PE injections, but this difference was not statistically significant (P = 0.11). Baroreceptor afferent activity hit a nadir with the first dose of SNP in pregnant animals.
and returned to that level with each succeeding dose (Fig. 7B). The relationships between HR and MAP were also similar between groups during the acute pressure changes evoked by bolus injections of PE (pregnant = \(-46 \pm 0.10\), virgin = \(-0.40 \pm 0.18\)) or SNP (pregnant = \(-0.11 \pm 0.09\), virgin = \(-0.13 \pm 0.08\); data not shown).

**DISCUSSION**

This study compared baroreceptor afferent discharge in response to increases and decreases in arterial pressure in pregnant and virgin rats. We found that the slopes of the relationships between ADNA and MAP during constant infusions of PE and SNP were attenuated in pregnant rats because of a more rapid adaptation of baroreceptors to a pressure stimulus. This contrasted with the similar ADNA response to acute pressure changes during bolus administration of the two agonists. Adaptation, or resetting, of baroreceptors in response to changes in prevailing arterial pressure is well known (6, 23, 29). Adaptation involves a shift in the operating set point of the receptor to levels that are closer to the new prevailing pressure and may also involve changes in the sensitivity or gain of the baroreceptor reflex. For example, during chronic hypertension, baroreceptors reset to a higher pressure and baroreflex responses are attenuated compared with the control state (6). Adaptation of baroreceptors to increases and decreases in pressure is known to be rapid, occurring within minutes to a few hours of exposure to changes in arterial pressure (5, 29). Thus, the chronic reduction in MAP during pregnancy most likely results in resetting of the baroreceptors toward lower prevailing pressures. This concept is supported by evidence of shifts in the midpoint of baroreflex curves for control of renal sympathetic nerve activity (RSNA) (11) and HR (26) to lower operating pressures in pregnant rats. The increases in MAP evoked by PE infusion in the present study did not raise ADNA to a maximum in virgin rats; therefore, analysis of the curve midpoint could not be compared with that in pregnant rats. Linear regression analysis of either end of the baroreflex curve, however, revealed significantly different y-intercepts, indicating a left shift in the baroreceptor response to increases or decreases in pressure. Thus it appears that the shift in baroreceptor activity during pregnancy parallels the shift in reflex responses to baroreceptor activation that have been reported (3, 11, 26).

Whether baroreflex sensitivity (gain) is altered by pregnancy has been a subject of debate. Baroreflex control, primarily of HR during hypertension, has been...
reported to be increased (10), decreased (3, 27), or unchanged (26). Indeed, our own study reported no differential effects of sinoaortic denervation on pressor responses to several vasoconstrictors in pregnant compared with virgin rats (18). These disparate findings may relate to use of anesthetized or conscious preparations, species differences, vasoactive agonists used, and methods of analysis. In contrast to the varying reports of baroreflex responses to hypertension, baroreflex control of HR and sympathetic nerve activity during hypotension has been more consistently shown to be attenuated in the pregnant animal (2, 11, 21, 26).

In the present study, baroreceptor activity in response to decreases in blood pressure was clearly shifted to the left, and the slope of this relationship, when plotted as a function of the percent change in MAP, was significantly blunted. Thus the reduction in baroreceptor afferent input in the pregnant rat supports the findings of blunted efferent reflex effects during hypotension. The curve relating ADNA to MAP during increases in pressure in pregnant rats intersected the curve in virgin rats, suggesting that the level of pressure attained may influence the comparison between groups. This observation could help to explain some of the divergent reports of baroreflex sensitivity during increases in pressure; that is, depending on the range of pressure evoked, baroreceptor reflex responses could appear augmented, unchanged, or blunted. When ADNA was plotted as a function of the percent change in MAP during PE infusions, however, a significant attenuation of the curve was observed, as has been noted at the low-pressure end of the curve as well. Brooks et al. (3) have concluded that examination of the entire baroreflex curve, that is, reflex responses to loading and unloading of baroreceptors, indicates an overall blunting of baroreflex control during pregnancy, and our findings confirm this hypothesis.

Changes in HR were measured in this study to assess reflex effects of baroreceptor stimulation. In anesthetized pregnant rats, HR was essentially unchanged in response to the range of pressures evoked by PE or SNP infusions. The lack of reflex change in HR is consistent with blunting of afferent baroreceptor input and/or faster adaptation to a pressure stimulus in this group, but it is unclear why there was no HR change during changes in arterial pressure. Pregnant rats had baseline HR that were relatively high, particularly during SNP infusions, and this may have affected the ability to increase HR; that is, baseline HR may have been close to maximum. The range of pressures evoked by PE and SNP infusions was also limited in pregnant animals and may not have provided an effective stimulus. Although a relatively low dose of pentobarbital sodium (35 mg/kg) was used in this study, there may have been a differential effect of anesthesia on reflex responses in pregnant vs. virgin rats. It is clear that HR does change in response to pressure changes in pregnant animals, since there was measurable bradycardia in response to acute changes in pressure with PE. Reflex tachycardia during SNP injections was minimal in both groups, again suggesting that the resting levels were close to the maximal plateau. Further studies will be needed to correlate reflex changes in sympathetic outflow with baroreceptor afferent discharge to develop a more complete picture of changes in baroreflex gain during pregnancy.

Bolus administration of PE and SNP was employed to compare the effects of acute changes in pressure with the more sustained (10 min/dose) changes evoked by constant infusions. In contrast to the attenuation of slopes relating ADNA and MAP in pregnant rats during constant infusions, the ADNA/MAP correlation during bolus injections was similar in both groups. During bolus SNP injections, ADNA in pregnant but not in virgin rats hit a nadir immediately in response to the first decrease in MAP and plateaued at that level in response to all other higher doses. Thus, although presumably baroreceptors reset to operate nearer the lower arterial pressure of pregnancy, it may be that the new operating point is close to the limit of the receptors’ ability to respond to hypotension. This possibility would contribute to the more rapid decline in MAP during hypertensive hemorrhage (3) and the smaller increases in sympathetic nerve activity during hypotension (11, 26) that have been reported. The gain of baroreceptor responses to acute changes in pressure, however, was not altered in pregnant compared with virgin rats, suggesting similarly effective buffering of transient pressure changes, at least within the range of pressures evoked in this study. When an alteration in pressure was maintained for even 10 min, however, baroreceptors in pregnant rats rapidly adapted, rendering the animal less able to compensate for changes in blood pressure. This pattern of baroreceptor adaptation at high pressures could lead to the further reduction in baroreflex sensitivity that has been observed in severe preeclampsia (1) and could contribute to the sustained vasoconstriction characteristic of this disease state.

Mechanisms that modulate baroreceptor activity include mechanical factors related to stretch or distortion of the nerve ending, as well as humoral factors, such as endothelial cell-derived substances and circulating hormones (4, 7, 28). Nitric oxide (NO) is known to suppress baroreceptor activity (7), and pregnancy is associated with increased synthesis of NO (9). Thus prolonged exposure to a pressure change could possibly stimulate increased release of NO in the aortic arch and attenuate baroreceptor firing. Pregnant rats and humans are also hyponatremic (25), and the reduction in extracellular sodium concentration could play a role in attenuated baroreceptor discharge, as was reported by Thoren et al. (30) in nonmyelinated baroreceptor afferent fibers from nonpregnant rats. The relatively long (50 min) exposure to changes in arterial blood pressure likely activated hormones such as angiotensin II and vasopressin. These peptides are known to modulate the baroreflex centrally (15, 31), and we cannot rule out a differential effect on baroreceptor afferent discharge in the two groups. These possibilities will require further investigation. There is evidence that aortic compliance is increased in normal human pregnancy (16). Presumably this mechanical adaptation would lead to in-
creased stretch of baroreceptor nerve endings in the aorta, thus increased baroreceptor firing. Whether this observation extends to the rat aorta during pregnancy is not known, but, if so, this would most likely be associated with increased rather than decreased baroreceptor activity. Plasma levels of estrogen hormones rise steadily throughout pregnancy in the rat (14), and 17β-estradiol administration to male rats has been shown to augment baroreflex sensitivity (28). If estrogen is influencing the baroreflex in the late-pregnant rat, however, it does not appear to be enhancing activity at the level of the peripheral baroreceptor. Although mechanisms for alterations in baroreceptor activity remain to be elucidated, it does appear that during pregnancy afferent information from baroreceptors regarding sustained changes in pressure is reduced at extremes of arterial pressure. The blunted afferent signal presumably would result in an attenuated ability to buffer changes in pressure and could contribute to findings, such as the more rapid drop in MAP during hemorrhage in pregnant dogs (2), or to the higher incidence of postural hypotension in pregnant women (20, 22). How baroreceptor afferent input is processed centrally could also influence reflex effects in the pregnant animal, and there is evidence that elevated levels of progesterone metabolites, known to be increased during pregnancy, may modulate baroreflex activity at brainstem sites (24, 26).

In summary, the aortic baroreceptor response to sustained increases and decreases in arterial pressure is attenuated in late-pregnant compared with virgin rats. This attenuation involves a more rapid adaptation to the pressure stimulus in the gravid animal. In addition, baroreceptor activity in the pregnant rat appears to be shifted toward the lower arterial pressure of pregnancy and may be operating close to the minimum firing level. Baroreceptor firing during acute pressure changes in pregnant rats is similar to that in the nonpregnant state, suggesting no change in the ability to buffer transient changes in pressure during pregnancy. How these gestational alterations in baroreceptor discharge are transduced and integrated centrally will need to be further defined; however, the findings of the present study suggest that attenuated afferent baroreceptor activity in pregnant compared with virgin rats contributes significantly to blunted baroreflex effects during pregnancy.

Perspectives

Many reports in experimental animals (2, 3, 10, 11, 17, 21, 26) and humans (13, 20, 22) have described alterations in baroreflex activity during pregnancy. Indeed, it appears that devastating conditions, such as preeclampsia, may be associated with exaggerated changes in baroreflex function (1). Alterations in baroreflex responses during pregnancy could be due to changes in the afferent, central integration sites, or effector targets. It is known that the responsiveness of a primary effector target, vascular smooth muscle, is attenuated during pregnancy (8, 12), and there is also evidence implicating central integration sites in baroreflex changes (17, 26). This is the first report of gestational attenuation of the afferent limb of the baroreflex. The important new finding is that aortic baroreceptors in the pregnant rat adapt more rapidly to a sustained pressure stimulus. The reduction in afferent input during pregnancy could contribute to attenuation of reflex effects, particularly those reported in response to unloading of baroreceptors (2, 3, 11, 26), and could play a role in conditions, such as supine and orthostatic hypotension in the pregnant human (20, 22). We have recently presented preliminary data describing attenuation of activity in cardiac receptors in pregnant rats in response to increases in right atrial pressure (19). Those receptors were also rapidly adapting. Thus, it may be that there is a global gestational effect on autonomic regulation that includes all components of the reflex arc. The new findings of attenuated afferent discharge imply that studies of the central and efferent mechanisms of autonomic regulation during pregnancy will have to take into account differential activity in afferent receptors.

This work was supported by National Institutes of Health Grant NR-04184.

Address for reprint requests and other correspondence: T. Hines, Univ. of Pittsburgh School of Nursing, 440 Victoria Bldg., Pittsburgh, PA 15261 (E-mail: thine@pitt.edu).

Received 8 July 1999; accepted in final form 13 January 2000.

REFERENCES