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Research Article

The objectives of this study were to determine if psychosocial stress impairs dilation through endothelium-derived relaxing factor (EDRF)-mediated mechanisms and if this effect is long lasting. Monkeys were fed an atherogenic diet for 36 mo while in one of three experimental conditions: (a) stable social groups ("unstressed," n = 6); (b) unstable social groups for the first half of the experiment and stable groups for the second half ("early stress," n = 8); and (c) stable groups for the first half of the experiment and unstable groups for the second half ("late stress," n = 6). Iliac arteries were studied in organ chambers containing Krebs' buffer and 10^{-6} M indomethacin. Arteries from the late stress group had impaired dilation (shift of the dose-response curve down and to the right) to acetylcholine and the calcium ionophore A23187 (for both, $P < 0.05$), but not to nitroprusside ($P > 0.05$), compared with unstressed or early stress monkeys. NG-methyl-L-arginine reduced the dose-response curve to both acetylcholine and A23187 in the unstressed group and resulted in similar vascular responses among all three groups ($P > 0.05$). We conclude that current, but not previous, exposure to chronic stress impairs endothelium-mediated dilation of atherosclerotic iliac arteries of cynomolgus monkeys through an EDRF-mediated mechanism.

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Effects of Psychosocial Stress on Endothelium-mediated Dilation of Atherosclerotic Arteries in Cynomolgus Monkeys

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Abstract

The objectives of this study were to determine if psychosocial stress impairs dilation through endothelium-derived relaxing factor (EDRF)-mediated mechanisms and if this effect is long lasting. Monkeys were fed an atherogenic diet for 36 mo while in one of three experimental conditions: (a) stable social groups ("unstressed," $n = 6$); (b) unstable social groups for the first half of the experiment and stable groups for the second half ("early stress," $n = 8$); and (c) stable groups for the first half of the experiment and unstable groups for the second half ("late stress," $n = 6$). Iliac arteries were studied in organ chambers containing Krebs' buffer and 10^{-6} M indomethacin. Arteries from the late stress group had impaired dilation (shift of the dose-response curve down and to the right) to acetylcholine and the calcium ionophore A23187 (for both, $P < 0.05$), but not to nitroprusside ($P > 0.05$), compared with unstressed or early stress monkeys. N^G -methyl-L-arginine reduced the dose-response curve to both acetylcholine and A23187 in the unstressed group and resulted in similar vascular responses among all three groups ($P > 0.05$). We conclude that current, but not previous, exposure to chronic stress impairs endothelium-mediated dilation of atherosclerotic iliac arteries of cynomolgus monkeys through an EDRF-mediated mechanism. (*J. Clin. Invest.* 1993. 92:1819-1823.) Key words: atherosclerosis • endothelium-derived relaxing factor • *Macaca fascicularis* • psychological stress • vasomotor system

Introduction

Patients with coronary artery atherosclerosis are prone to the development of vasospasm, particularly at sites of coronary artery stenosis (1). Vasospasm, in turn, may contribute to the pathogenesis of myocardial ischemia and infarction (1-3). It has been shown in both human beings and monkeys that atherosclerosis may predispose arteries to vasospasm by impairing endothelium-mediated dilation and augmenting constriction of arteries (4-6).

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Coronary artery reactivity, however, is affected by factors other than the extent of atherosclerosis. For instance, acute stress in people (7) and chronic stress in monkeys (8) impair endothelium-mediated dilation of coronary arteries. The effect of stress on vasomotion is not surprising, as mental stress in patients with coronary artery disease can trigger transient myocardial ischemia and regional flow disturbances (9, 10).

In previous studies, acetylcholine was used to test endothelium-mediated dilation of coronary arteries (4, 7, 8). It remains unclear, however, if stress impairs endothelium-mediated dilation indirectly through alteration of muscarinic receptors or directly through endothelium-derived relaxing factor (EDRF)-mediated mechanisms. Furthermore, it is unknown if the effects of stress on vascular reactivity can be reversed by removing the stress.

The current study was designed as an interventional trial where cynomolgus monkeys (*Macaca fascicularis*) fed an atherosclerotic diet were housed in one of three conditions: (a) stable social groups (no psychosocial stress); (b) unstable social groups during the first half of the experiment and stable social groups during the second half of the experiment (early psychosocial stress), or (c) stable social groups during the first half of the experiment, but disruption of social groups during the second half of the experiment (late psychosocial stress). The study's three objectives were to determine: (a) the effects of psychosocial stress on endothelium-mediated dilation of atherosclerotic arteries; (b) if the effect of stress on endothelium-mediated dilation is mediated through EDRF activity; and (c) if previous psychosocial stress (early stress) has a residual effect on endothelium-mediated dilation of atherosclerotic arteries.

Methods

Experimental design. 20 adult male cynomolgus monkeys (Charles River Research Breeding Laboratories, Inc., Wilmington, MA) were used in this study. The 20 monkeys were a part of a larger study involving 75 monkeys. During a 5-mo preexperimental period, all 75 monkeys were fed a moderately atherogenic diet containing 0.25 mg of cholesterol per kilocalorie and consisting of 18% of calories from protein, 43% from lipid, and 39% from carbohydrate. The primary source of cholesterol was dried egg yolk. At the end of this preexperimental period, monkeys were divided into three experimental conditions and balanced for behavioral and plasma lipid (total plasma cholesterol [TPC] and HDL cholesterol) characteristics. All monkeys were housed in social groups of five animals each throughout the experiment. The three experimental conditions were as follows:

(a) The monkeys in the "unstressed" condition ($n = 25$) were fed the atherogenic diet throughout the experimental phase (36 mo) and were housed in social groups of unvarying, stable membership.

1. Abbreviations used in this paper: EDRF, endothelium-derived relaxing factor; TPC, total plasma cholesterol.

(b) The monkeys in the "early stress" condition ($n = 25$) were fed the atherogenic diet throughout the experiment and underwent the stress of monthly reorganization of social group membership during the first half (18 mo) of the experimental phase. Monkeys in this condition were housed in stable social groups the second half of the experiment.

(c) The monkeys in the "late stress" condition ($n = 30$) were fed the atherogenic diet throughout the experiment, were housed in stable social groups for the first half of the experiment (18 mo), and underwent the stress of monthly social reorganization during the last half (18 mo) of the experimental phase.

Vascular responses were determined in randomly chosen monkeys from the three treatment conditions (unstressed, $n = 6$; early stress, $n = 6$; late stress, $n = 8$).

Social stress manipulation. Macaque monkeys are highly aggressive primates that live together successfully by virtue of elaborate social mechanisms and relationships. Animals within groups establish among themselves hierarchies of relative dominance, in which some monkeys habitually win fights or contests while others habitually and predictably lose. In addition, networks of affiliation and coalition tend to link animals together in mutually beneficial ways and temper, in part, the actions of more aggressive monkeys. A characteristic of particular usefulness to researchers is that the introduction of unfamiliar monkeys to each other occasions considerable emotional perturbation and aggressive interaction as animals attempt to reestablish generalized hierarchic relationships and affiliative coalitions.

In previous studies as well as the current experiment, the group membership of the psychosocially "stressed" monkeys was altered at monthly intervals by redistributing animals among social groups (within each experimental category) such that each monkey was housed with four different monkeys after each reorganization. We have shown previously that a monthly schedule of social distribution alters affiliative and aggressive behavior (11, 12), impairs endothelium-mediated dilation of coronary arteries (8), and significantly exacerbates coronary artery atherosclerosis (13). Monkeys housed in stable social groups did not undergo redistribution of group membership. The behavioral repertoire of macaques living in small groups has been described by ourselves and others (11–14). The social behavior of all animals was recorded three times per week in 30-min sessions using a laptop computer (Tandy TRS 80, model 100; Radio Shack, Fort Worth, TX) (15). During these samples, technicians recorded all incidents of fighting, play, grooming, and passive body contact. Technicians also recorded the activities of all animals at predetermined times, allowing estimates to be made of the percentage of time each animal engaged in particular behaviors. Observations were balanced across experimental conditions and for the time of day of sampling.

Cardiovascular and blood parameters. Venous blood samples were obtained and blood pressure and heart rates measured from sedated monkeys (ketamine hydrochloride, 10–15 mg/kg intramuscularly) at 3-mo intervals. The monkeys were sampled in the morning after an overnight fast. Values reported in this study were determined from the blood sample taken at the time of angiography. TPC was measured by the methods of Allain et al. (16) and HDL cholesterol by standardized methods (17). Blood pressure and heart rate determinations were measured with a Dinamap Research monitor (1245 or 1255; Critikon, Tampa, FL). This method has been validated for indirect measurements of blood pressure and heart rate in cynomolgus monkeys (18).

Vessel bath experiments. Vascular responses were measured using the common iliac artery removed from the monkeys before necropsy. The left common iliac artery was identified and carefully dissected from connective tissue. The artery segment was removed and placed in cold Krebs' buffer for transport to the laboratory. Approximately 25 min elapsed between removal of the artery segment and mounting of the ring segments in the vessel bath. Six ring segments (2 mm in diameter) were cut from one common iliac artery and mounted in a 25-ml bath containing Krebs' buffer solution equilibrated with 95% oxygen and 5% carbon dioxide and temperature controlled at 38°C. The techniques and apparatus used to measure vascular responses of monkey

iliac arteries using vessel baths have been described previously (19). All vessels were studied in the presence of 10^{-6} M indomethacin to prevent activation of the cyclooxygenase system. The resting length of each segment was adjusted so that tension development to 100 mM potassium chloride was optimized.

Measurement of vascular responses. After preconstriction from 1 to 3 μ m with prostaglandin $F_{2-\alpha}$, dilation (defined as percent reduction in precontracted tension) was measured in response to receptor-mediated release of nitric oxide (acetylcholine 10^{-9} – 10^{-4} M), non-receptor-mediated release of nitric oxide (the calcium ionophore A23187 10^{-10} – 10^{-6} M), and endothelium-independent dilation (nitroprusside 10^{-9} – 10^{-4} M). Dilator responses to acetylcholine and A23187 were measured before and 40 min after addition of blockade of nitric oxide synthase (N^G -methyl-L-arginine 10^{-5} M).

Measurements of coronary artery atherosclerosis. Monkeys were euthanized with sodium pentobarbital (80 mg/kg intravenously) after removal of the iliac artery used for vascular responsiveness studies. The cardiovascular system was then flushed with normal saline and perfused with 10% neutral buffered formalin at a pressure of 100 mmHg for 1 h. The heart was immersion fixed in 10% neutral buffered formalin. Morphometric analysis of atherosclerosis extent was obtained from the left anterior descending coronary artery (morphometric data were not available for iliac arteries). Five serial tissue blocks were cut at approximately 3-mm intervals and perpendicular to the long axis of the coronary artery. Histological sections were stained with Verhoeff-van Gieson's stain. These sections were projected, and cross-sectional area of plaque lesion was measured with a digitizer. Atherosclerosis extent was expressed as the mean cross-sectional area of the intima in square millimeters.

Statistical analyses. Values shown are mean \pm SEM. Data distributed nonnormally were first subjected to linear or square root transformation. To determine whether psychosocial status influenced dilator responses of iliac arteries, data were evaluated by repeated-measures ANOVA. To determine whether psychosocial factors influenced TPC, HDL cholesterol, heart rate, and blood pressure, data were subjected to a series of one-way (Treatment^{UNSTRESSED, EARLY STRESS, LATE STRESS}) ANOVAs. Post hoc analyses of data were carried out using Duncan's multiple comparison procedure. The Kruskal-Wallis test was used to analyze the behavioral data, with the z -test statistic used for pairwise comparisons.

Results

Plasma lipids, heart rate, blood pressure, and atherosclerosis extent. There were no significant effects of treatment (early or late stress) on TPC ($F = 0.567$), HDL cholesterol ($F = 0.425$), resting blood pressure ($F = 0.610$), resting heart rate ($F = 0.715$), or intimal area ($F = 0.988$) (for all, $P > 0.25$) (Table I).

Social behavior. Animals in the early and late stress conditions exhibited significantly higher rates of contact aggression

Table I. Cardiovascular Parameters in Each Experimental Group (mean \pm SEM)

| Groups | TPC | HDLC | Heart rate | Blood pressure | Intimal area |
|--------------|--------------|------------|-------------|----------------|-----------------|
| | mg/dl | mg/dl | bpm | mmHg | mm ² |
| Unstressed | 474 \pm 61 | 45 \pm 8 | 145 \pm 4 | 71 \pm 3 | 0.45 \pm 0.2 |
| Early stress | 534 \pm 37 | 40 \pm 5 | 144 \pm 4 | 69 \pm 6 | 0.44 \pm 0.14 |
| Late stress | 525 \pm 27 | 52 \pm 8 | 145 \pm 8 | 65 \pm 3 | 0.72 \pm 0.16 |

bpm = beats per minute; intimal area (mm²) = plaque extent in coronary artery, in square millimeters.

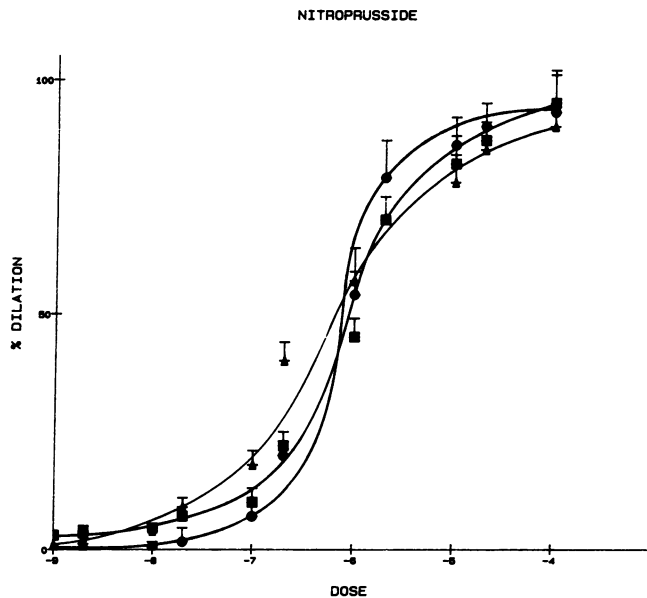


Figure 1. Effect of treatment with nitroprusside, in a vessel bath preparation, on percent dilation of iliac arteries in cynomolgus monkeys from three experimental groups: unstressed controls (triangles), early stress (squares), and late stress (circles). Refer to text for definition of stress groupings.

and spent more time alone anxiously scanning the environment compared with the animals in the unstressed condition (z test, $P < 0.005$). Their behavioral differences between conditions (stress, unstressed) were only observed during the period

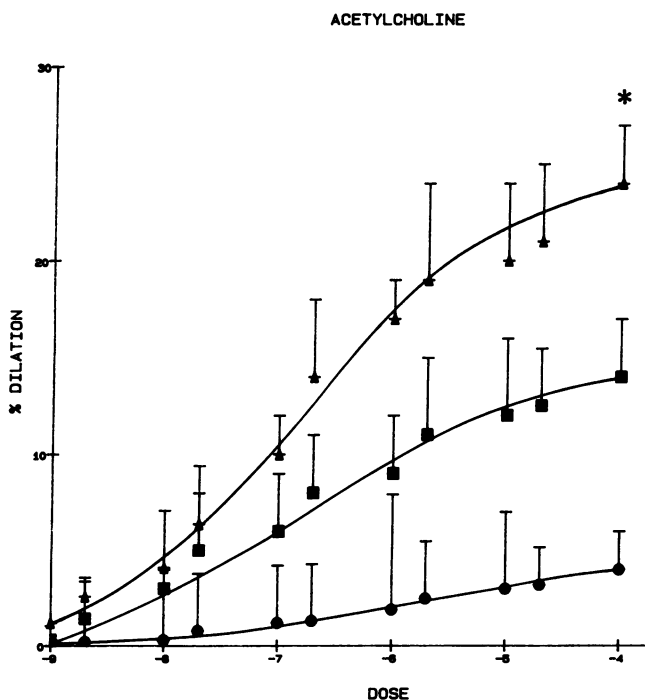


Figure 2. Effect of treatment with acetylcholine, in a vessel bath preparation, on percent dilation of iliac arteries in cynomolgus monkeys from three experimental groups: unstressed controls (triangles), early stress (squares), and late stress (circles). Refer to text for definition of stress groupings.

of chronic stress (i.e., after removal of the stress, behavior of the monkeys in the stress condition was similar to that observed in the unstressed condition).

Vascular responses. There was no effect of treatment (early or late stress) on dilator response to nitroprusside among groups of monkeys ($F = 0.356$, $P = 0.22$, Fig. 1). There was, however, an effect of treatment on dilator responses to acetylcholine ($F = 3.54$, $P = 0.03$, Fig. 2) and A23187 ($F = 4.41$, $P = 0.0001$, Fig. 3). Post hoc analysis revealed that the vascular responses to acetylcholine and A23187 were similar in arteries from monkeys in the late stress and unstressed groups ($P > 0.05$). However, arteries from monkeys in the late stress groups had impaired endothelium-mediated dilation (shifted dose-response curve down and to the right) compared with the other two groups ($P < 0.05$). Incubation of artery segments with N^G -methyl-L-arginine eliminated significant treatment effects on vascular response to acetylcholine ($F = 0.892$, $P = 0.36$, Fig. 4) and A23187 ($F = 0.340$, $P = 0.72$, Fig. 5). N^G -methyl-L-arginine inhibited dilation to acetylcholine and A23187 in the unstressed condition ($P < 0.05$), but not in the early or late stress conditions ($P > 0.05$).

Discussion

The three major findings of this study were that (a) psychosocial stress impairs endothelium-mediated dilation through receptor (acetylcholine)- and non-receptor-mediated (A23187) mechanisms; (b) the effect of stress on endothelium-mediated dilation is mediated through EDRF mechanisms, since it occurs in the presence of both indomethacin and A23187, but is blocked by N^G -methyl-L-arginine; and (c) endothelium-mediated dilation is impaired by concomitant, but not previous, chronic stress.

Results of the present experiment support those of previous experiments demonstrating that psychosocial stress impairs en-

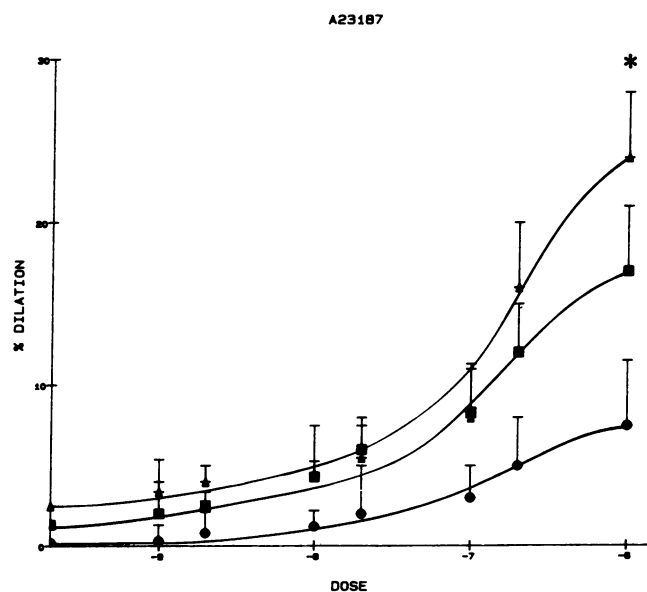


Figure 3. Effect of treatment with the calcium ionophore A23187, in a vessel bath preparation, on percent dilation of iliac arteries in cynomolgus monkeys from three experimental groups: unstressed controls (triangles), early stress (squares), and late stress (circles). Refer to text for definition of stress groupings.

ACETYLCHOLINE + LNMMA

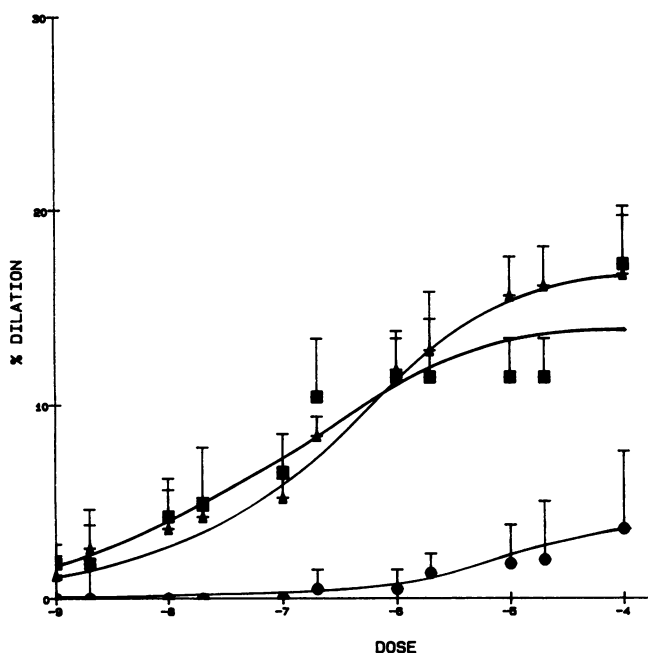


Figure 4. Effect of treatment with acetylcholine plus N^G -methyl-L-arginine (LNMMA), in a vessel bath preparation, on percent dilation of iliac arteries in cynomolgus monkeys from three experimental groups: unstressed controls (*triangles*), early stress (*squares*), and late stress (*circles*). Refer to text for definition of stress groupings.

endothelium-mediated dilation both of coronary arteries of monkeys during regression of atherosclerosis (8) and of patients with suspected coronary heart disease (7). In these studies, the effects of psychosocial stress on endothelium-mediated dilation were tested with intracoronary infusions of acetylcholine, which activates muscarinic receptors and the release of EDRF (20, 21). Results of the present study extend the findings of previous studies by showing that the effects of psychosocial stress on endothelium-mediated dilation: (a) are not dependent on muscarinic receptors, because A23187 has the same effect; (b) are mediated through EDRF mechanisms, since effects are inhibited by N^G -methyl-L-arginine; and (c) are not mediated through products produced by the cyclooxygenase pathway, because stress effects occurred in the presence of indomethacin. Furthermore, these data suggest that previous stress does not have lasting effects on endothelium-mediated dilation, as monkeys in the early stress condition had endothelium-mediated dilator responses similar to those of the unstressed group.

The mechanisms by which psychosocial stress impairs EDRF activity remain unclear. Results of the present experiment indicate that plasma lipids and atherosclerotic plaque size are not the determining factors that alter vascular responsiveness; however, the numbers of animals in each group were small. Larger numbers may, or may not, have determined whether the trend toward more atherosclerotic plaque in the late stress condition was significant. If so, then plaque extent may have been a determinant of vascular reactivity.

Social disruption of monkeys in the short term causes a modest increase in heart rate and damage to the vascular endothelium, both of which can be reduced by blockade of β -adren-

ergic receptors (22). Monkeys housed in unstable social groups may have repeated acute episodes of sympathetic stimulation during confrontations with other animals. Similarly, significant increases in catecholamine secretions, blood pressure, and heart rate occur during laboratory-induced mental stress (23, 24). Repeated episodes of acute sympathetic stimulation result in sharp increases in blood pressure and heart rate, which may damage the vascular endothelium and impair the release or augment the breakdown of EDRF.

Results of previous studies indicate that EDRF production in atherosclerotic arteries is similar to that of normal arteries (25). On the other hand, breakdown of EDRF is augmented in atherosclerotic arteries, possibly by exposure of EDRF to oxygen radicals (26, 27). It is unclear from the present study if psychosocial stress impairs EDRF activity through decreased release or increased breakdown of EDRF. However, preliminary data indicate that shear stress (which is altered during episodes of increased sympathetic arousal) changes EDRF production (28). Therefore, it is reasonable to speculate that chronic stress may downregulate EDRF production. This hypothesis is consistent with our observation that current, but not previous, stress impairs endothelium-mediated arterial dilation.

Limitations of the study. The present experiment used iliac arteries studied in vessel baths, whereas previous experiments have used coronary arteries studied in vivo (7, 8). The effect of stress on vascular responses may differ at various arterial sites because of different hemodynamics and arterial receptor number and function. However, the effect of stress on endothelial damage is similar in coronary and iliac arteries (22). Furthermore, in the current study, the effect of stress on endothelium-mediated dilation in iliac arteries was receptor independent. Therefore, it is reasonable to speculate that similar results

A23187 + LNMMA

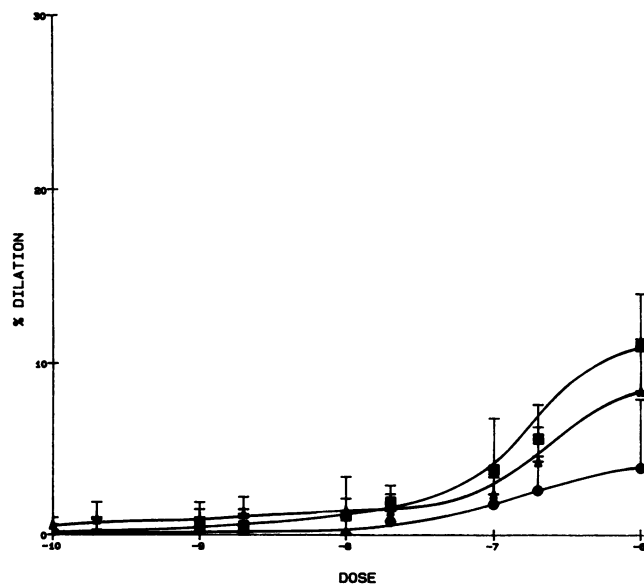


Figure 5. Effect of treatment with the calcium ionophore A23187 plus N^G -methyl-L-arginine (LNMMA), in a vessel bath preparation, on percent dilation of iliac arteries in cynomolgus monkeys from three experimental groups: unstressed controls (*triangles*), early stress (*squares*), and late stress (*circles*). Refer to text for definition of stress groupings.

would have occurred if coronary arteries had been used in the present experiment.

Conclusions. Results of the present study indicate that psychosocial stress impairs EDRF activity and that previous stress may not have a lasting effect on vascular reactivity. Such data may be of considerable importance in helping to resolve the current uncertainty regarding the role of psychosocial stress as an independent risk factor on the pathogenesis of coronary heart disease in human beings.

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