Experimental Studies

Pravastatin Protection from Cold Stress in Myocardium of Rats

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SUMMARY

The aim of this research was to evaluate the possible protective effect of pravastatin on ultrastructural alterations induced by cold stress in the myocardium of rats.

Sixteen EPM-Wistar rats (Rattus norvegicus albinus) were used and distributed into four groups: 1) control; 2) pravastatin; 3) cold stress, and 4) pravastatin + cold stress. A daily oral dose of 10 mg/kg of weight of pravastatin was administered to each rat in groups 2 and 4 for 15 days. The stress induced by cold was obtained by keeping the group 3 and 4 rats in a freezer at -8°C for 4 hours. The animals were killed and the heart and fragments of the left ventricles (LV) were removed and processed prior to conducting electron microscopic analysis.

The ultrastructural alterations in cardiomyocytes were quantified through the number of mitochondrial cristae pattern (cristalysis). The group subjected only to cold stress showed a significant increase in cristalysis (391.9) when compared with control group (42.0). In the cold stress and pravastatin pretreatment group, a statistically significant (96.9)*, $P<0.05$ cristalysis reduction was observed when compared with cold stress group. The mitochondrial cristalysis profiles of the control and pravastatin groups were 42.0 and 65.7, respectively.

Cold stress induced a significant increase in the rate of mitochondrial cristalysis. In the group that received pravastatin and was exposed to cold stress, the drug protected the LV cardiomyocytes. This fact was confirmed by a reduction mitochondrial cristalysis pattern. (Jpn Heart J 2003; 44: 243-255)

Key words: Cold stress, Pravastatin, Cardiac mitochondria

STRESS is often defined as a threat, real or implied, to homeostasis and this term refers to the maintenance of a narrow range of vital physiological parameters necessary for survival.

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Stress represents the effort performed by an organism to adapt or react against factors that threaten to break the internal steady state.[1)] More than 60 years ago, Selye recognized that the physiological system was activated by stress, and that protecting and restoring the body could also damage the system.[2)]

The physiological response to stress, that is to say the load, must be understood in terms of a process and not as a static, isolated, and independent reaction, since the moment in which it begins a prolonged biochemical process occurs independently of the cause of tension. There are various stressor agents, for example, physical, chemical, biological or psychological factors, but they always elicit similar mechanisms of adaptation.[1,2)]

Allostasis,[3)] the ability to achieve stability face to changes, as the process for actively maintaining homeostasis, using the autonomic nervous system (ANS), the hypothalamo-pituitary-adrenal (HPA), the cardiovascular, metabolic, and immunological systems to protects the organism against internal and external stresses.[3)]

The allostatic response begins with an increase in circulating catecholamines from the ANS and glucocorticoids from the adrenal glands. This adaptative process begins through intracellular receptors for steroid hormones, plasma membrane receptors, and secondary messengers for catecholamines. The maintenance of the adaptative process can alter the structure and function of a variety of cells and tissues.[4,5)]

Ultrastructural analysis of mitochondria is very important, and as an organelle which is extremely sensitive to hypoxia, it represents a structure of central importance in aerobic metabolism.[6)] Mitochondria are the powerhouses of the cell where oxidative phosphorylation takes place to generate ATP. Oxidative phosphorylation involves the transfer of electrons to oxygen coupled to the synthesis of ATP. Oxidative stress is the increased generation of free radicals resulting in oxidative damage to DNA, proteins, and lipids. Impairment of mitochondrial oxidative phosphorylation is associated with increased oxidative stress.[7)]

The ultrastructural changes in the cardiomyocytes of rats subjected to cold stress are characterized by myofilament dearrangement, increasing the gap between mitochondrial cristae sometimes causes erasings, and partial or total rupture of cristae originating in lacular areas.[8)]

A study in a rat model in which hypoxia was produced through coronary artery occlusion described focal lesions similar to those described above, pointing to an acute myocardial hypoxic state produced by the increase in oxygen consumption.[9)]

The loss of cellular membrane stabilization during hypoxia affects aerobic respiration, causing a reduction in ATP molecule production and consequently

activating anaerobic glucolysis, a process which determines lactic acid and inorganic phosphate accumulation and decreases intracellular pH. The decrease in ATP compromises the Na^+ pump mechanism, an energy-dependent process, with consequent intracellular Na^+ retention and intracellular edema. Diverse effects have been verified secondary to cold-stress exposure, ie, an increase in catecholamine liberation, an increase in the relative disproportion of intracellular calcium, hypoxia, and ATP depletion act in conjuction in the pathogenesis of ultrastructural alterations induced in cardiomyocytes.[10)]

In clinical trials dedicated to the primary[11)] and secondary[12-14)] prevention of coronary heart disease, it was demonstrated that pravastatin modified the actuarial survival curves; after a myocardial infarct the treated group early changes from the control group. Based on this fact, it was suggested that pravastatin had a rapid onset protective effect opposed to a slow effect secondary to an action against progression of the occlusive atherosclerotic plaque process in coronary arteries.[12)] The present study was conducted to investigate other possible mechanisms involved in the early protection due to this drug. Since pravastatin is a very common drug in clinical medicine, the main objective was to evaluate its possible protective effect against myocardial injury induced by cold.

MATERIALS AND METHODS

Sixteen adult male EPM-Wistar rats weighing between 250-300 g were used. The animals were kept in plastic and metal cages. The room temperature was kept stable at 22°C, and a circadian rhythm was maintained unaltered. The animals received food and water *ad libitum*. After a one week adaptation period, the animals were selected randomly and assigned to four groups of four animals each:

Control (CON): received 1 mL of water *per os* by gastric gavage for 15 consecutive days and were kept at normal room temperature.

Pravastatin (PR): received 1 mL of a pravastatin solution (10 mg/kg of weight) for 15 consecutive days and were kept at normal room temperature.

Stress (ST): received 1 mL of water by gastric gavage for 15 consecutive days prior to an experimental procedure, and were kept in a freezer for 4 hours at -8C°.

Pravastatin+stress (PR+ST): received 1 mL of a pravastatin solution identical to the PR group for 15 consecutive days preceding the experiment and were kept in a freezer for 4 hours at -8°C.

Immediately after light ether anesthesia, the thorax of each rat was opened and the heart was exposed and removed. Cold 2% gluteraldehyde solution was dropped on the still beating heart. Fragments of the left ventricle wall were then removed and fixed in phosphate buffered glutaraldehyde at pH 7.2. The frag-

ments were cut into small 1 mm cubic pieces which were postfixed in 1% OsO_4 solution for 2 hours at 4C° and then dehydrated and embedded in araldite. Silver or gray thin sections (60-90 mm) were selected and cut on a Porter-Blum MT-B ultramicrotome, mounted on copper silver grids with 200 patches, and stained with uranyl acetate and lead citrate. The samples were selected, collected, and deposed on copper wire with 200 patches. The preparations were examined with an electronic microscope (Model EM 90, Carl Zeiss) using a tension of 80 kV.

All electromicrographs were taken under the same magnification parameters, and their dimensions at the end of the process were 18×24 cm (final amplification × 8,800). The mitochondrial profiles, including the integrity, of the electron micrographs were analyzed. Mitochondrial lesions (cristalysis) were defined as a partial or complete lysis of cristae and their substitution by lacunar areas.

Six electromicrographs of LV fragments, which were analysed by 3 observers independently with the same and standardized criteria, were obtained randomly from each group of examined animals.

Twenty other rats were distributed into four equal groups according to the same criteria. Total cholesterol (C) and creatine phosphokinase (CPK) levels in serum were measured.

Statistical analysis: The following tests were used. 1) Analysis of variance with Kruskal-Wallis[15] test to compare the groups among themselves in relation to the variable: C and CPK levels, number of mitochondria with cristalysis; when any difference was detected among the groups a test of multiple comparisons was performed.[16] 2) To analyse the concordance of measurements performed by three observers, the index of Bartko for intraclass correlation was used according to the Fleiss guidelines.[17] This test uses the following formula:

$$R = \frac{N\,(PMST - EMS)}{N.\,PMS + (K-1)\,RMS + (N-1)(K-1)\,SEM}$$

Where,
PMS = Patients mean square
RMS = Researcher mean square
EMS = Error mean square
N = number of observed events
and,
K = number of observers.

The formula describes the variability which can occur in each measurement performed, between the observers, and also the variability due to random error. When R is higher than 0.75, the concordance between the observers is excellent, independent of the statistical significance of the results.

The level of significance was 5%.

RESULTS

The transmission electron microscopy results for the CON group showed myocardium fibers and/or elongated and branched cardiomyocytes surrounded by sarcolemma. There was generally only one nucleus inside the cell usually located in the central portion of the fiber. In the sarcoplasm, high concentrations of mitocondria were observed. Sarcomeres, limited by 2 Z-bands, are composed of two types of myofilaments; a gross filament composed of myosin, and a thin filament composed of actin. They are contained in fascicules called myofibrils. The mitochondria were dispersed in the sarcoplasma or interposed among abundant myofibrils, characterized by a large number of cristae (Figure 1).

In the pravastatin group (PR), the cardiomyocytes exhibited a pattern similar to that of the group of cells belonging to the control group (CON) (Figure 2).

The stress group (ST) cardiac fibers presented areas of myofibrils well preserved, with a structural pattern similar to nomal, and numerous areas with focal loss of myofibrils. A great number of mitochondria showed different degrees of

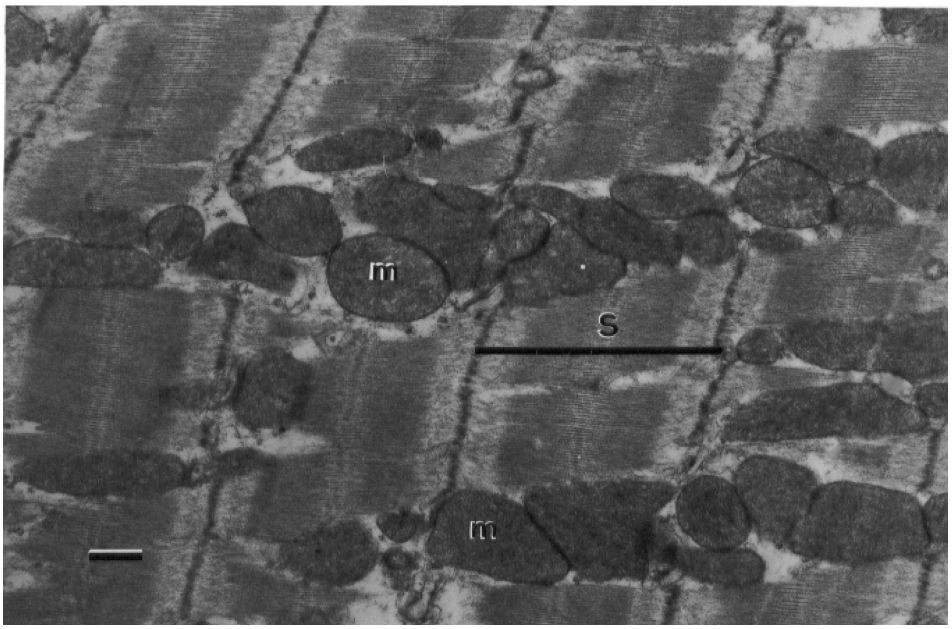

Figure 1. Electron micrograph of longitudinal slices of left ventricular cardiomyocytes of rats in the control group (CON). Note the increased mitochondrial concentration (m). Myofibrils have been cut logitudinally where it is noted the sarcomeres (S) are delineated by their Z-lines (Bar = 0.5 μm).

alterations such as swelling, and decrease in number as well disruption of the cristae with formation of large vesicles (Figure 3).

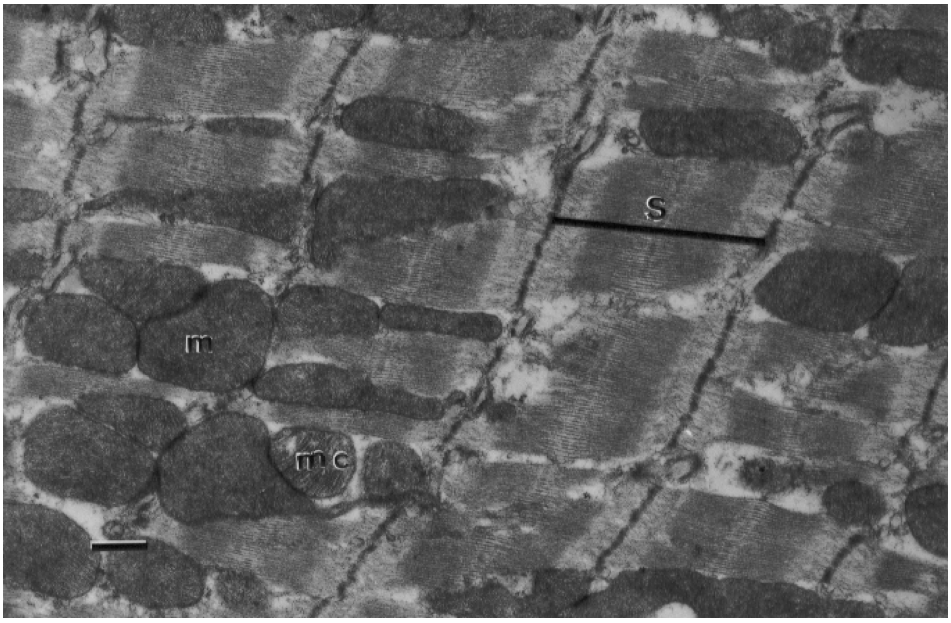

Figure 2. Electron micrograph of longitudinal slices of left ventricular cardiomyocytes of rats in the pravastatin group (PR). Observe the increased concentration of intact mitochondria (m) and rare mitochondria with initial cristalysis (mc). The sarcomeres (S) are well delineated by Z lines (Bar = 0.5μm).

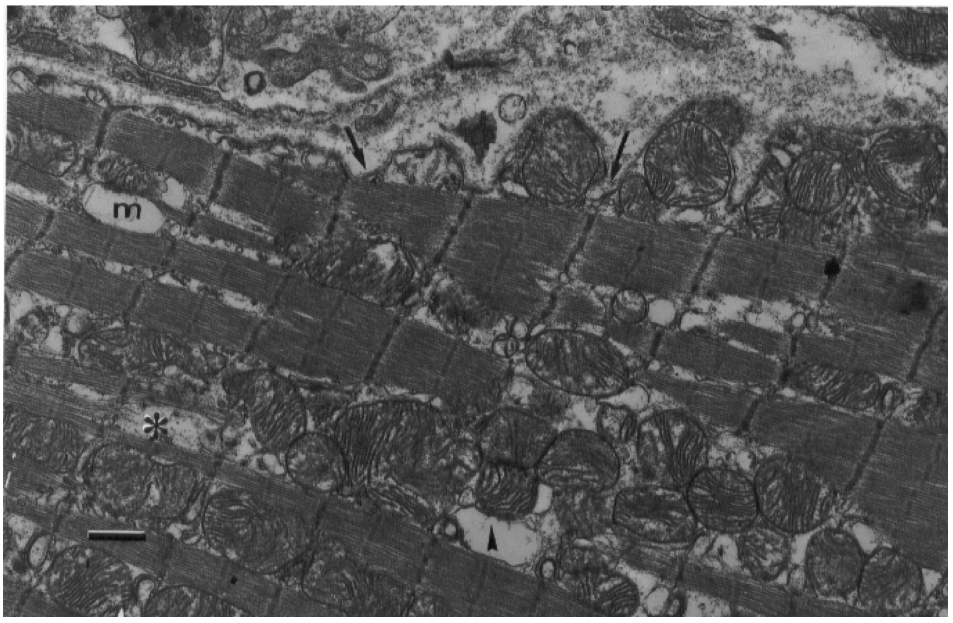

Figure 3. Electron micrograph of longitudinal slices of left ventricular cardiomyocytes of rats in cold stress group (ST). Note the increased concentration of mitochondrial lysis (m). The intercellular space between two cardiomyocytes is shown. The sarcoplasmic membrane shows a notched edge (↓). It is possible to observe some myofilaments with evident discontinuity (*). The head of the arrows (▲) shows the lacunar spaces (Bar = 0.8μm).

In the pravastatin + stress group (PR+ST), cardiomyocytes with intact cells were observed. The sarcoplasmic membrane, in the majority of images, had a continuous and rectilinear appearance. The sarcoplasma replete of mitochondria with varied forms and dimensions, distributed in an interposed manner between myofilaments. Some lipid droplets were also observed near mitochondrial conglomerates. The myofilaments were normally arranged with some areas of discontinuity, showing a uniform periodicity of sarcomeres. The nucleus, generally unique and central, showed a euchromatic pattern (Figure 4).

Morphometric analysis: The number and the mean of mitochondrial counting profile with crystalysis in the cardiomyocytes of rats belonging to the four groups were measured by three independent observers. The results are presented in Tables I and II. The high index of intraclass correlation (R) observed varied between 0.60 to 0.99, confirming the reliability of this method for determining the difference between the groups (Table II). Table II showed for each animal, the results of three observers, for considering the reproductibility.

Statistically significant differences among the groups were demonstrated and they were complemented by a test of the difference among the means. The ST group was statistically different compared to the CON and PR groups ($P < 0.05$).

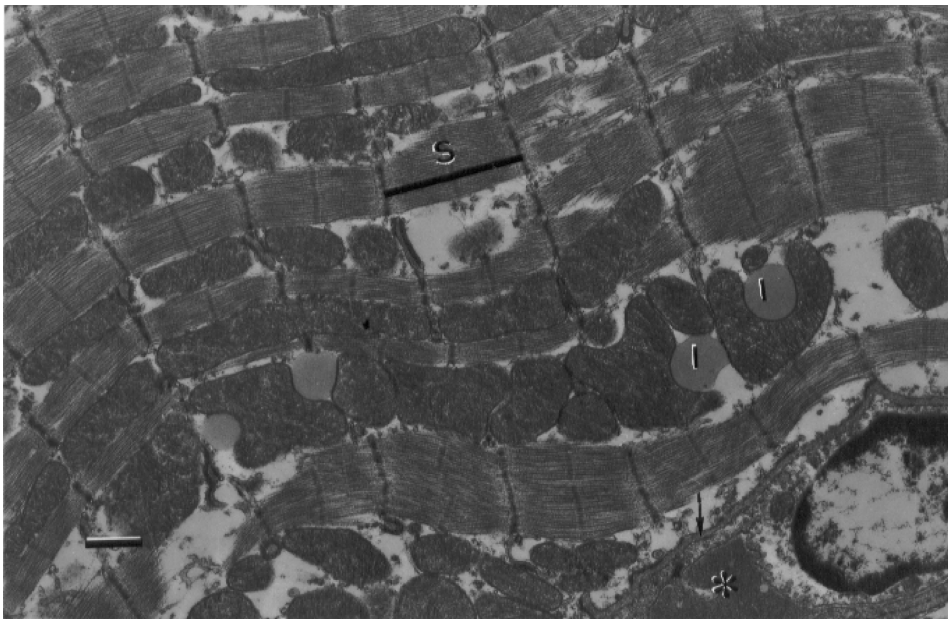

Figure 4. Electron micrograph of longitudinal slices of left ventricular cardiomyocytes of rats in the cold stress and pravastatin pretreatment group (PR + ST). Note partial view of two cardiomyocytes separated by a straight and intact sarcolemma (↓). The intercellular space occupied by a capillary, showing a euchromatic nucleus and a red blood cell (*). The sarcolemmal membrane is filled by a high concentration of mitochondria with some lipid droplets (I) and myofilaments forming well-defined sarcomeres (S) (Bar = 0.8μm).

Table I. Values of Mitochondrial Profiles with Crystalysis for Six Photomicrographs Analysed by Three Independent Observers (O1, O2, O3) in the Control (CON), Pravastatin (PR), Stress (ST), and Pravastatin + Stress (PR+ST) Groups

	Groups											
Numbers	CON			PR			ST			PR+ST		
	O1	O2	O3	O1	O2	O3	O1	O2	O3	O1	O2	O3
1	8	6	6	9	4	4	58	58	59	20	20	24
2	8	5	5	18	18	17	64	65	65	17	16	16
3	6	6	6	6	3	3	64	64	64	13	13	13
4	6	4	4	13	13	13	90	90	88	13	13	10
5	8	10	10	13	13	17	70	66	66	17	17	16
6	7	10	11	9	12	12	48	48	49	18	19	15
Total	43	41	42	68	63	66	394	391	391	98	98	94

CON, R = 0.60, PR, R = 0.80, ST, R = 0.99, PR + ST, R= 0.82.

Table II. Comparative Analysis of Mitochondrial Crystalysis Profile Number

	Groups			
Photos	CON	PR	ST	PR+ST
1	6.7	5.7	58.3	21.3
2	6.0	17.7	64.7	16.3
3	6.0	4.0	64.0	13.0
4	4.7	13.0	89.3	12.0
5	9.3	14.3	67.3	16.7
6	9.3	11.0	48.3	17.3
Mean	7.0	10.95	65.3	16.1

The values represented in the columns express the mean result between three independent observers in the control (CON), pravastatin (PR), stress (ST), and pravastatin + stress (PR + ST) groups. Variance analysis was performed using the Kruskal-Wallis method. Hc. = 7.82, Hcalc. = 17.61*, (*$P < 0.005$). Comparison of means: Zc. = 2.6 and Zcalc. = 10.77** (**$P < 0.05$). ES > CON; PR and PR + ST.

Table III. Total Cholesterol (C) Values and Creatine Phosphokinase (CPK) Enzyme Levels in the Control (CON), Pravastatin (PR), Stress (ST), and Pravastatin+Stress (PR + ST) Groups

	Groups ($n = 20$)			
	CON	PR	ST	PR+ST
C (mg/dL)	96±15	93±19	106±24	99±12
CPK (U/L)	1833,3±739	1522±374	1708±300	1078±314

C: $P > 0{,}05$-not significant; CPK: $P > 0{,}05$-not significant.

The PR+ST group showed intermediate values between the CON and PR groups, which was interpreted as partial protection of pravastatin during stress induced by cold (Table II).

The C and CPK levels among the groups were not statistically different (Table III).

DISCUSSION

In the present study, cold was used as an alarmogen agent and pravastatin as a pharmacological substance to verify possible myocardial protection. Pravastatin is a drug belonging to a group of competitive inhibitors of HMG-coA reductase, and this enzyme has been implicated in cholesterol synthesis.[18] It was observed in this experiment that pravastatin in the short term neither reduced cholesterol levels and nor provoked muscle damage as demonstrated by CPK measurements (Table III).

It was observed that pretreatment of the rats with pravastatin submitted to cold stress according to this methodology produced an evident direct protective effect on cardiomyocyte lesions demonstrated by mitochondrial ultrastructure preservation. This result indicates that the effect of pravastatin on cardiomyocyte protection is independent of cholesterol reduction since in the short term a reduction in cholesterol levels in the PR and PR+ST groups was not observed.

Different clinical trials have demonstrated significant reductions in cardiovascular events, coronary events, and death due to ischemic heart disease with the clinical use of HMG-coA reductase inhibitors such as pravastatin.[12,19-22]

Studies related to atherosclerosis regression in humans have reported different and contrasting results ranging from a large decrease in clinical events to a small reduction in the degree of coronary stenosis in coronary angiography studies. Physical regression of the atheroma may not be the main mechanism of reduction in cardiac risk.[23-26] This is probably due partially to a normalization of vasomotor function dependent on endothelial cells.[27-31]

Myocardial perfusion studies[32-34] and endothelial function studies[35,36] have shown clinical and functional improvements before the detection of lesional atherosclerotic regression. They also showed an improvement in vasodilatory function of vascular endothelium at the level of the coronary[30,37,38] and peripheral circulation[39] through the use of HMG-coA reductase inhibitors alone or in combination with antioxidant therapy.[29,40]

Hemostatic and inflammatory processes are possibly affected by pravastatin. It can reduce platelet aggregation activity by altering the cholesterol content of the platelet membrane which modifies its fluidity.[41] In this way, it can inhibit

the interaction between platelets and fibrinogen and reduce the expression of diverse agents involved in the coagulation process.[42)]

Pravastatin has anti-inflammatory effects, and promotes a blockade of macrophage activation[43)] and the formation of foam cells.[43,44)] More recently it was demonstrated that the drug promotes *in vitro* inhibition of PDGF and also affects the transduction intermediated by angiotensin II through the suppression of two proto-oncogenes, *c-jun* and *c-fos*,[45)] indicating the possibility of inducing a regression of myocardial hypertrophy through the blockade of independent signaling in the reduction of cholesterol.[46)]

The protection of cardiomyocytes by pravastatin in this study most likely occurred due to its action on the modulation of vascular relaxation mediated by the endothelium. This improvement of local regulation of coronary arterial tonus could be a factor in the ischemic process produced by stress. The clinical benefits associated with pravastatin not explained by low density lipoprotein cholesterol (LDL) reductions may be the result of an independent action of pravastatin on endothelial nitric oxide synthase (e NOS) activation.[47,48)] Pravastatin stimulates an increase in nitric oxide (NO) production by cultured endothelial cells similar to the effects seen with acetylcholine.[48)]

It has been suggested that increased plasma LDL inhibits the active transport of L-arginine (L-ARG) by endothelial cells,[49)] uncoupling the L-ARG: eNOS pathway and leading to superoxide anion production.[50)] Because both superoxide anion and its reaction product NO, and peroxynitrite produce tissue injury,[51)] it is possible that an indirect action of pravastatin may be present to normalize endothelial function by protecting the active arginine transport from injury by LDL, thereby preventing the formation of both superoxide anion and peroxynitrite.[50,51)]

These results in animals subjected to cold stress and treated with pravastatin showed a direct effect in addition to the global protection of cardiomyocytes. There was a statistically significant specific reduction of mitochondrial crystalysis, mainly related to the integrity of the mitochondria, since this was the parameter quantified to measure cardiomyocyte function during the exposure to cold. At some point in this chain of events, pravastatin produced an interference culminating in such biomolecular action.

The protection of cardiomyocytes subjected to cold stress under the experimental conditions used in this present work can be included in the effects of pravastatin. This knowledge may contribute to a better understanding of the feasibility of using this agent in therapies of diverse clinical conditions involving this kind of stressful situation.

These protective effects may be responsible for the clinical results observed in the survival curves of the CARE study.[12)]

These findings may possibly open new perspectives for more research and may benefit experimental and clinical investigations. The present results indicate the importance of pravastatin and its immediate effects on myocardial protection.

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