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Changes in the Coronary Vascular System Following Prolonged Exposure to Stress[1]

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BASSETT, J. R. AND K. D. CAIRNCROSS. *Changes in the coronary vascular system following prolonged exposure to stress.* PHARMAC. BIOCHEM. BEHAV. 6(3) 311–318, 1977. – Hearts of stressed rats showed marked changes in the coronary vasculature. It was suggested that such morphological changes could be explained on the basis of an increased coronary vascular permeability. Endogenous inflammatory substances could induce swelling of the endothelial cells and cause separation of the neighbouring cells from one another, thus allowing the passage of lipid molecules through the endothelial lining. In order to gain supportive evidence for the above hypotheses an electron microscope study was undertaken. The presence of junctional gaps in the endothelial lining of the coronary vascular system was observed following prolonged stress, as well as platelet aggregation. The use of lipid staining of frozen sections indicated the presence of large lipid deposits in the arteriole walls, corresponding to vacuoles seen previously. It would appear therefore, that prolonged exposure to stress may result in pathological changes in the myocardium associated with changes in the vascular endothelial permeability, and platelet aggregation. Pathological changes induced in this way, however, should be inhibited by high glucocorticoid levels and should not be manifested until adaptation of the steroid response to stress has occurred. Measurement of plasma glucocorticoid levels over the period of prolonged stress shows a good correlation between the adaptation of the steroid response and the onset of a progressive degeneration of the coronary vascular system.

Prolonged stress Junctional gaps Vascular permeability Platelet aggregation Lipid deposition Corticosterone

EXPOSURE to prolonged stress, in which the psychological parameters of anxiety or fear are prominent, appears to be associated with many facets of cardiovascular disease [4,22]. More recently Bassett and Cairncross [1] have shown that subjecting rats to an irregular, signalled foot-shock for up to 70 days will produce changes in the microcirculation of the heart. These changes included congestion and dilatation of the large venules, collecting venules and veins; an increase in PAS +ve material marginated in the endothelium of coronary vessels, and the presence of vacuoles in the media, adventitia of the arteriole wall. To explain such stress induced changes in the coronary vasculature it was postulated that exposure to stress had resulted in an increased vascular permeability. Endogenous inflammatory substances released in response to the stressor may cause neighbouring endothelial cells to separate, thus allowing the passage of large lipid molecules through the endothelial lining. In the compact tissue of the arteriole wall these lipid molecules would accumulate and appear as vacuoles. Bassett and Cairncross further postulated that the accumulation of PAS +ve material marginated in the endothelial lining, represented a thrombotic deposit or platelet aggregate. Such a response could contribute to the observed congestion and dilatation of the microcirculation.

In order to gain evidence in support of the above hypotheses it was decided to subject rats to a similar stress regimen and, with use of the electron microscope, to examine the coronary vasculature for the presence of junctional gaps in the endothelial lining, and evidence of platelet aggregation or thrombotic deposits. To confirm that the vacuoles seen previously in the arteriole walls were in fact lipid droplets, hearts from stressed animals were frozen sectioned and stained for light microscopy using a lipid stain.

METHOD

Animals

Male CSF rats 87–93 days old were used in all experiments. The animals were housed in groups of 3 under conditions of constant temperature and humidity (21 ± 0.5°C, 46% humidity) and subjected to a 12 hr night-day regimen (light 8 p.m.–8 a.m.) beginning at least 14 days prior to commencement of experimentation and continuing until its conclusion. Food and water were provided ad lib.

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Both control and stressed rats were housed under identical conditions.

Stress Apparatus

Animals were placed in automated 1-way avoidance boxes (Lafayette Model No. 85200). An escape platform was made available to the animal by an automated movable partition. A light conditioned stimulus (CS) of 2W was located on the wall of the grid chamber opposite to the escape platform. The unconditioned stimulus (UCS) was delivered by a generator-scrambler through the grids as a 2 mA, 50 pulses/sec square wave. Each rat was placed on the escape platform at the commencement of the treatment session. On each trial the CS onset 4 sec before the animal was pushed by the movable partition from the platform onto the grid which was simultaneous with the onset of the UCS. At this point in time the movable partition immediately retracted and the animal was able to jump from the grid to the reexposed platform with a minimum latency of 0.3 sec. The UCS was terminated by the return of the animal to the platform.

Stress Procedure

The stress treatment consisted of 7CS-UCS exposures randomly placed in a 35 min stress session. Each stress session was repeated once daily for periods of 1, 5, 10, 20, 40 and 60 days. For each time period the stress and control groups consisted of 12 animals. Stress treatments were carried out between the hours of 9 a.m.–12 noon.

Immediately following the completion of the last stress episode the animals were sacrificed by cervical dislocation and exsanguinated. Control animals were killed over the corresponding time periods. The blood was collected in heparinized tubes and centrifuged in order to obtain cell free plasma which was then frozen. Plasma corticosterone levels were determined subsequently by the fluorimetric method of Mattingley [20] which is specific for free 11-hydroxycorticosteroids.

For each time period 6 stressed and 6 control hearts were prepared for electron microscopy, and 6 stressed and 6 control hearts were prepared for frozen sectioning.

Electron Microscopy

Sections of the left ventricular wall were rapidly removed and fixed in 4% glutaraldehyde in phosphate buffer (pH 7.3) for 2 hr at 5°C. The tissues were then post-fixed in 1% osmium tetroxide for 1 hour, dehydrated and embedded in an epon-araldite mixture. Silver sections were cut and mounted on formvar coated copper grids. The sections were stained using 5% uranyl acetate saturated in 50% ethanol for 20 min, followed by Reynald's lead citrate solution for 20 min. The grids were examined in a Hitachi HS 8 electron microscope.

For each control and stressed heart a total of 35 blood vessels were examined. No distinction was made as to whether the vessels were arterioles, capillaries or components of the venous system. Due to the fact that capillaries were more numerous these vessels would contribute the greatest number of vessels examined. The vessels were examined for the presence of junctional gaps in the endothelial lining, and the number and form of platelets visible in the lumen.

Frozen Sections

Hearts were fixed in 10% formal saline for 7 days then embedded in 25% gelatine, 1% phenol. Using a cryostat, frozen sections (10μ thick) were cut transversely through the heart commencing at the apex. The sections were stained with Oil red-O in isopropanol, which is a general lipid stain staining neutral fats. The sections were examined for the presence of lipid deposits within the myocardium and coronary vascular system. Epicardial fat pads were included in some of the frozen sections in order to compare the colour and general appearance of lipid material using this stain.

RESULTS

Junctional Gaps

Junctions between endothelial cells of the rat coronary vascular tree could be grouped into a number of categories. The least complex form but also the most rarely seen was the situation where the cells met end to end, butting on to one another. By far the most common junctions however, were junctions where either there was an extensive overlap between adjacent cells or where there was an intrusion of a tongue from one cell into the groove formed by the other cell (Fig. 1). Both junctional types occurred with equal frequency. Occasionally adjacent endothelial cells would differ in staining density, one appearing light the other dark. Similar types of endothelial junctions and density of staining have been reported for rat aorta by Schwartz and Benditt [26]. In all cases the junctions appeared to be sealed. In some cases the junctions were similar to those described by Friend and Gilula [7] and Huttner, Boutet and More [13] as tight junctions. In other junctions the seal took the form of a number of focal strictures similar in appearance to a series of desmosomes (macula adherens) either on their own or together with tight junctions. Numerous plasmalemmal vesicles bounded by typical unit membranes were observed free on the cytoplasm or still in continuity with either the luminal or interstitial membranes. Similar vesicles have been reported previously in coronary endothelial cells, and it is suggested that large molecules may be transported exclusively by such vesicles [3, 13, 16]. Fenestrations were never observed. In the present study very little difference was apparent between the endothelial lining of the coronary arterioles, capillaries and venules. Bruns and Palade [3] found no consistent variation in rat coronary capillary endothelia from the arterial and venous ends of the vessels, and Rhobin [23] found no variation throughout the venous channels.

Following exposure of rats to stress no apparent changes were observed in the endothelial cells throughout the coronary vasculature with the exception of the appearance of junctional gaps in excess of 250 nm (Figs. 2 and 3). Junctional gaps were first observed following 20 days of stress but were most apparent following 40 and 60 days of stress. In the 60 day stressed animals a total of 10 junctional gaps were observed, gaps being present in 5 out of the 6 hearts examined; in the 40 day stressed animals a total of 7 gaps were observed, present in 3 out of the 6 hearts examined. In the 20 day stressed animals a single gap was observed. In all cases the junctional gaps were associated with an intact basement membrane. No junctional gaps were observed in any of the control hearts nor in the 1, 5 and 10 day stressed animals, despite the fact that the same number of vessels were examined.

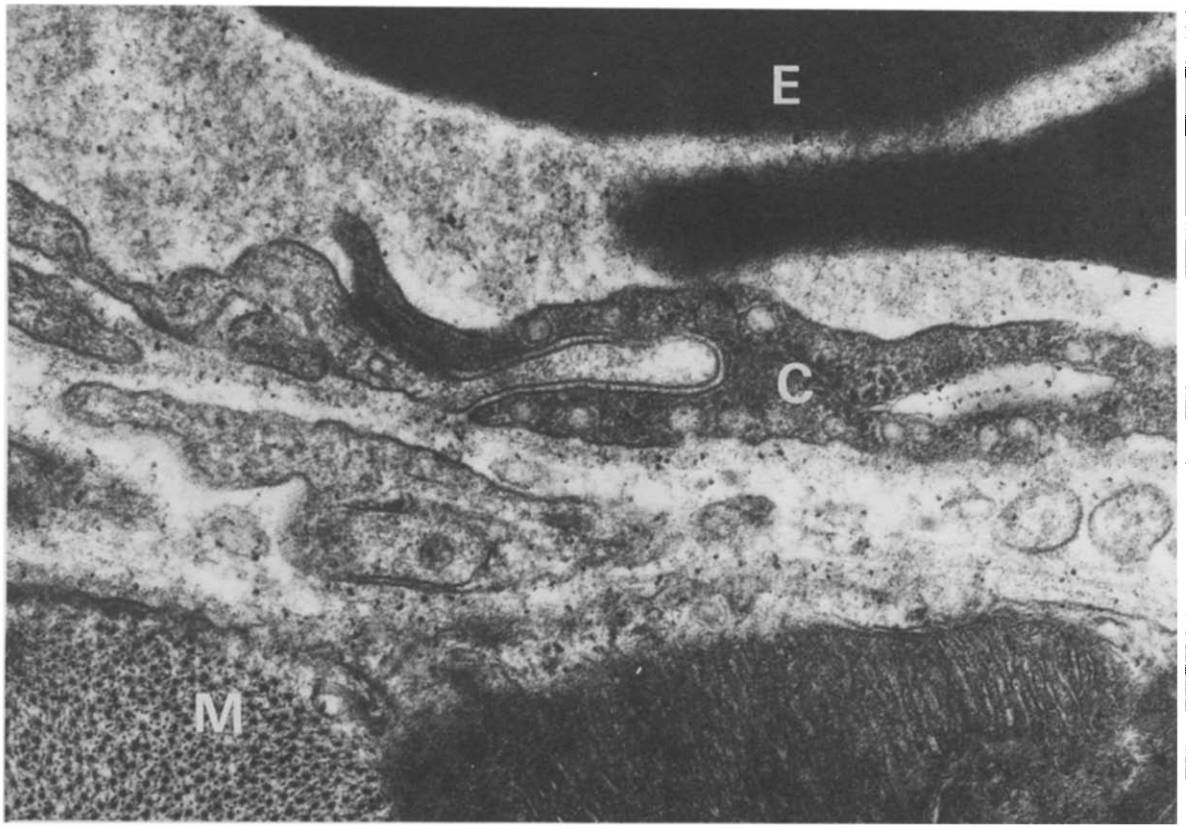

FIG. 1. Typical junction between two capillary endothelial cells in left ventricular wall of a control heart. C = endothelial cell; E = erythrocyte; M = cardiac muscle. (× 40,500).

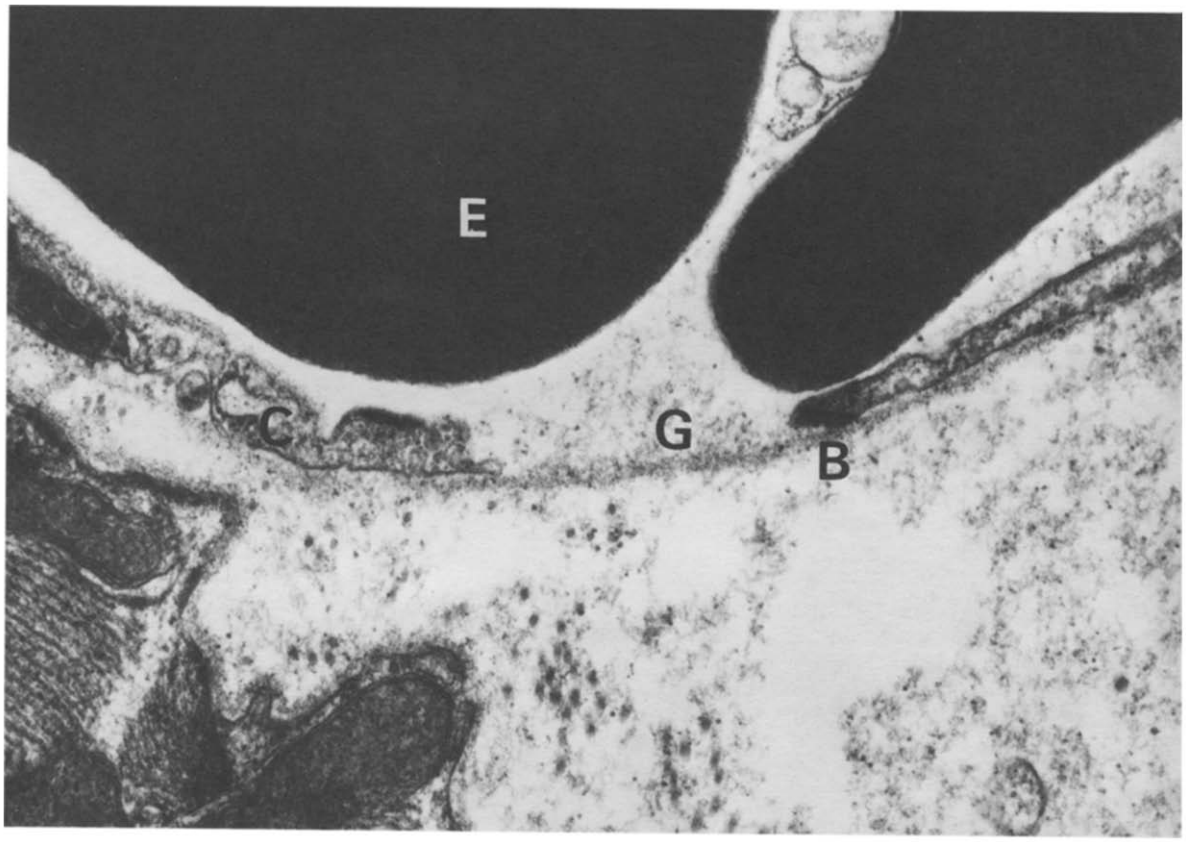

FIG. 2. Junctional gaps in the endothelial lining of capillaries from 60 day stressed hearts. E = erythrocyte; G = junctional gap; B = basement membrane; C = endothelial cell (× 39,000).

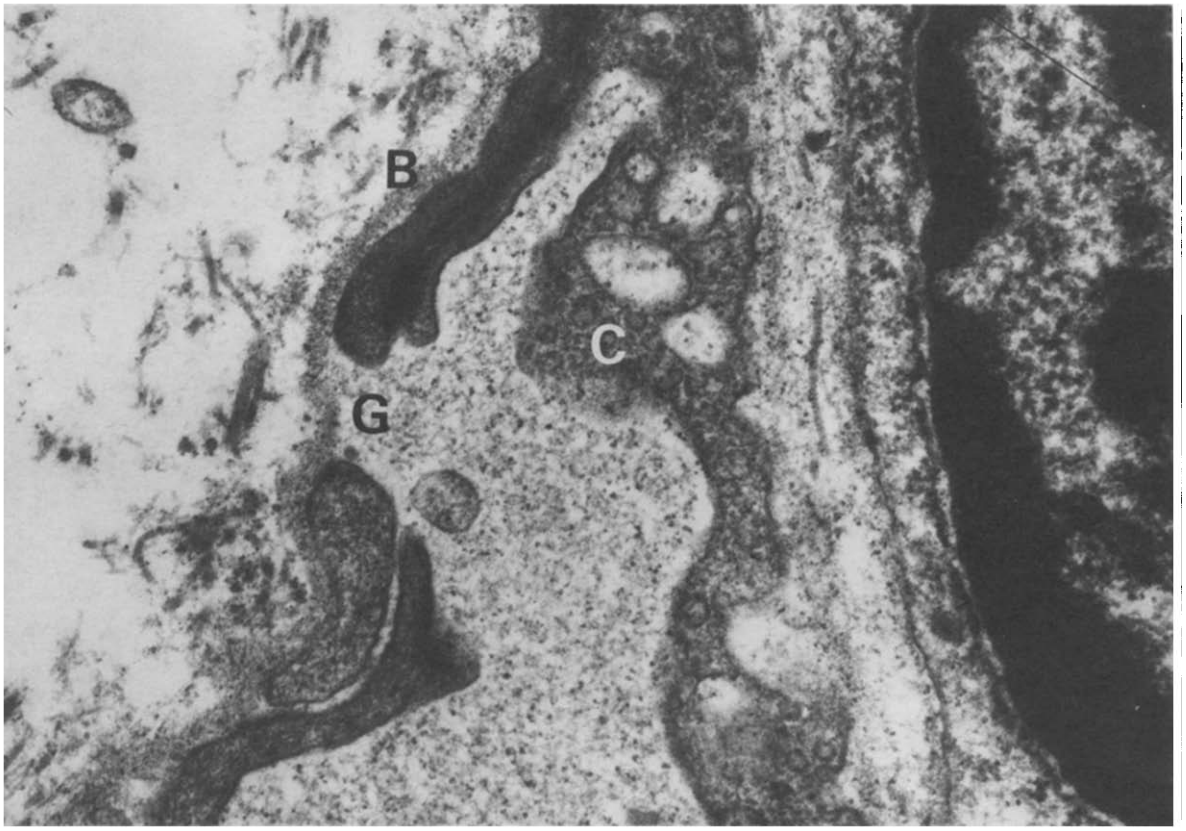

FIG. 3. See Fig. 2 legend.

Platelets

A count of the number of platelets visible in an equal number of coronary vessels showed a significant increase in both the 40 and 60 day stressed animals when compared to their corresponding control groups. The mean number of platelets visible in coronary vessels/heart examined (± S.D.) were: – 60 control 1.7 ± 0.8, stressed 4.2 ± 2.9 (t-test $p<0.02$); 40 day, control 0.8 ± 1.2, stressed 4.2 ± 2.4 (t-test $p<0.02$). There was no significant increase in any of the other stressed groups. In control animals the platelets were generally ovoid in shape and always seen midstream, away from the endothelial lining. Platelets in both the 40 and 60 day stressed animals were seen adhering to the endothelial lining (Fig. 4) as well as showing visible signs of aggregation. Aggregation was apparent by the loss of microtubules and ovoid shape with the development of pseudopodia (Fig. 5). No evidence however, was seen of fibrin deposition.

Frozen Sections

Frozen sections of hearts from 40 and 60 day stressed animals, stained with the lipid stain Oil red-O, showed the presence of large stained droplets in the media, adventitia of coronary arterioles (Fig. 6). Such stained deposits were similar in appearance to the lipid droplets present in the epicardial adipose tissue, and were similar in position and frequency to the vacuoles reported previously by Bassett and Cairncross [1]. No stained droplets were observed in the arterioles of the corresponding non-stressed control groups.

Plasma Corticosterone

The plasma corticosterone levels are shown in Table 1. The initial elevation to 90 μg/100 ml plasma is in close agreement with that reported by Bassett, Cairncross & King [2] using the same stress regimen. The initial steroid elevation was maintained unchanged over 5 days of stress before gradually falling to establish a new level of 44 μg/100 ml plasma by day 40. This new level of steroid elevation was subsequently maintained up to 60 days of stress. In all stressed groups the level of plasma corticosterone was significantly greater than that of their corresponding control group (t-test $p<0.01$ in all cases).

TABLE 1

MEAN PLASMA CORTICOSTERONE LEVELS (± SE)

Days of Stress	Corticosterone (μg/100 ml plasma) Control	Stressed	t-test $p<$
1	21.1 ± 2.6	90.2 ± 2.6	0.001
5	33.5 ± 2.9	90.3 ± 6.4	0.001
10	24.7 ± 3.1	76.5 ± 2.9	0.001
20	23.3 ± 1.8	68.0 ± 4.2	0.001
40	17.3 ± 2.0	43.6 ± 3.6	0.001
60	22.7 ± 2.4	42.4 ± 3.4	0.001

Number of animals/group = 12.

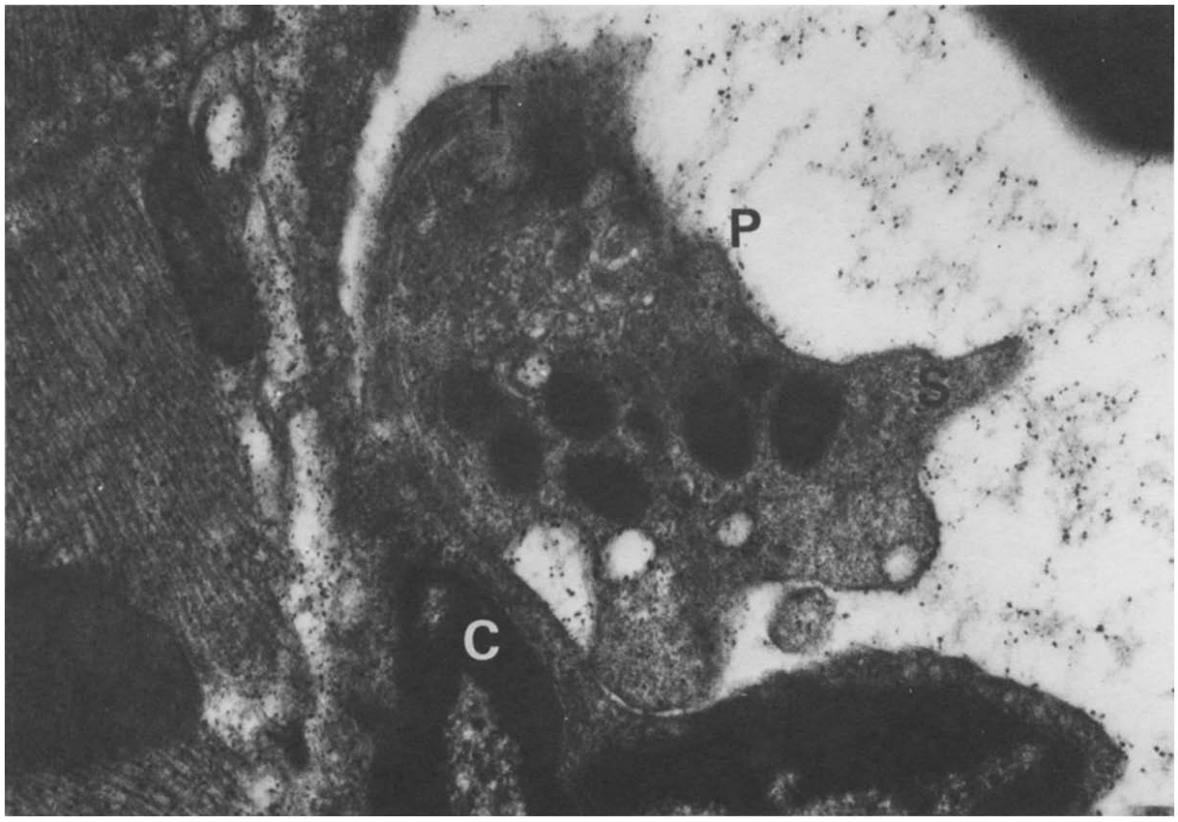

FIG. 4. Platelet adhering to the endothelial lining of a capillary from a 60 day stressed heart. C = endothelial cell; P = platelet; T = microtubules (not seen on right hand side due to development of pseudopodia (S)) (× 28,500).

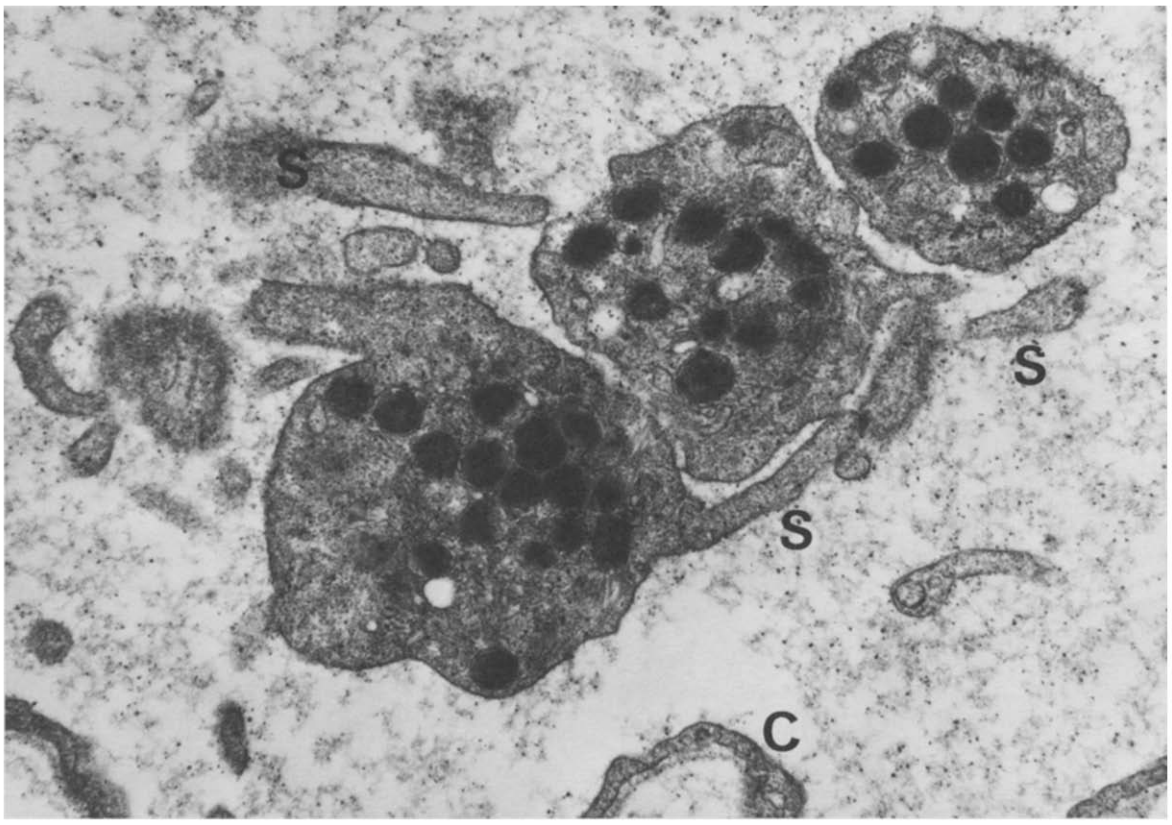

FIG. 5. Platelets aggregating in the lumen of a capillary from a 60 day stressed heart. Pseudopodia (S) can be clearly seen, with the loss of microtubules. C = endothelial cell. (× 20,000).

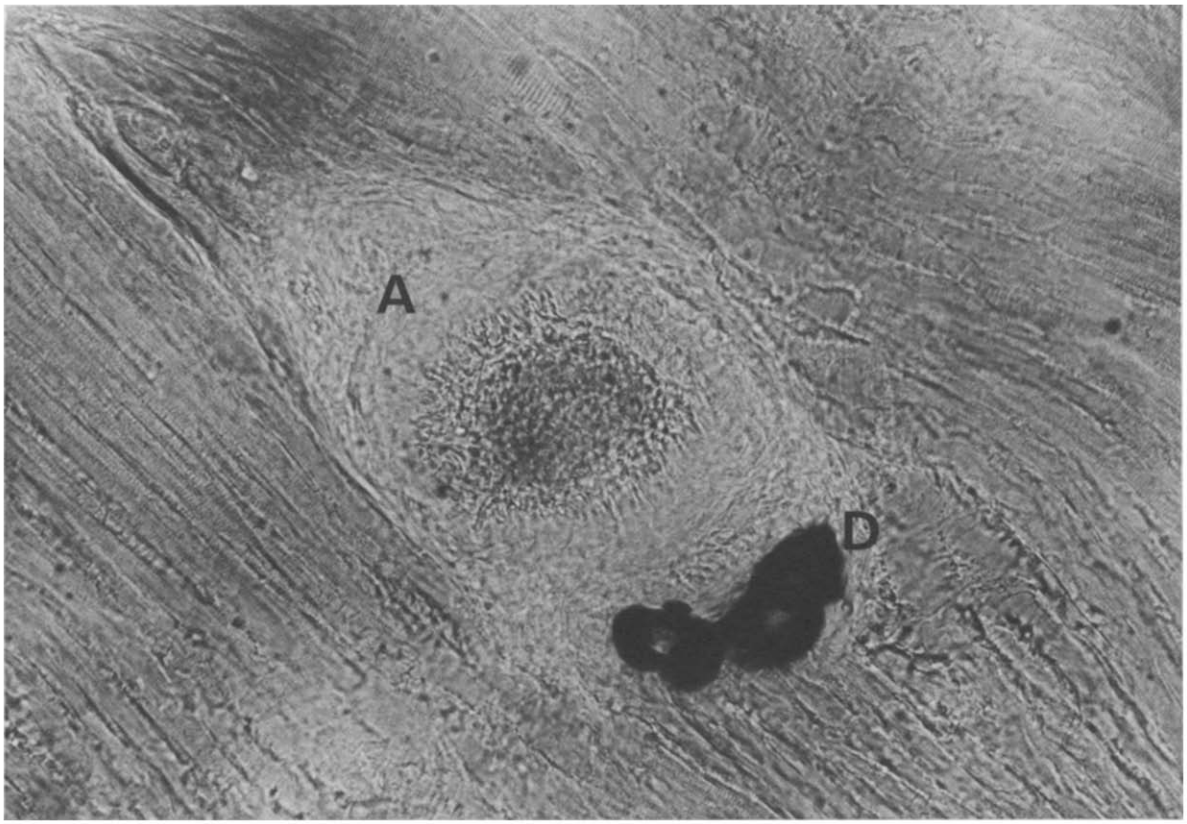

FIG. 6. Frozen section of left ventricular wall of 60 day stressed rat showing arteriole (A) with lipid stained droplets (D) in the adventitia (× 160).

DISCUSSION

Passage of large molecules through endothelial cells is generally confined to vesicular transport rather than passage between endothelial cells [16,26]. The intact endothelium provides a barrier against the free exchange of large lipid molecules, such as cholesterol, between the plasma and the extracellular fluid. It is only when the endothelium is damaged that the levels of such molecules equilibrate rapidly between the arterial wall and plasma [28]. Bassett and Cairncross [1] proposed that in situations of prolonged stress endogenous inflammatory substances such as histamine, serotonin and bradykinin can cause separation of neighbouring endothelial cells. In rat scrotum, histamine, serotonin and bradykinin have all been shown to increase vascular leakage by the opening of such endothelial gaps [17]. the proposal of stress induced endothelial cell separation is supported by the finding of junctional gaps in the hearts from 40 and 60 day stressed animals. The opening of such endothelial gaps would allow the passage of lipid molecules and cholesterol through the endothelial lining, where they would accumulate in the compact tissue of the media and adventitia of vascular wall. In the present study, using lipid stains, an accumulation of lipid material in these regions was apparent, supporting the assumption made by Bassett and Cairncross [1] that the vacuoles they observed were lipid deposits. Such lipid accumulation would be aggravated by the marked elevation in circulating free fatty acids [6] and myocardial triglycerides [18] that occur following exposure to stressful situations, and by the depression of myocardial oxidation of fatty acids reported by Jönsson and Johansson [15] following stress induced hypoxia.

The source of the endogenous inflammatory substances involved in the enhanced coronary vascular permeability may well be the suggested increase in mast cells reported to occur following prolonged exposure to stress [1]. Mast cells are found almost exclusively in connective tissue, particularly around small blood vessels; relatively few being found in the larger blood vessels [14], and are known to hold and release vasoactive substances, in particular histamine and serotonin [9]. The release of histamine may be triggered by changes in noradrenaline concentration [12], noradrenaline being released by the activation of the sympathetic-adrenal medullary axis in response to the stressor [19]. Another possible source of histamine may be an intrinsic histamine stored within the cells of the vascular system itself. Such a non-mast cell histamine was identified by Schayer [25] as being involved in microcirculatory changes.

The supposition that the PAS +ve deposits marginated in the coronary vasculature of stressed animals, may represent platelet aggregation has been substantiated by the findings reported in this study. Development of pseudopodia, apparent loss of microtubules, as well as adhesion of platelets to the endothelial lining, are all phenomena associated with the platelet aggregation. Surface activation and aggregation of blood platelets are two of the earliest changes in the formation of most kinds of thrombosis [24], although in the present study no fibrin formation was

apparent. As was the case with release of inflammatory substances platelet aggregation may result from stress induced stimulation of the sympathetic-adrenal medullary axis. Haft, Kranz, Albert and Fani [11] found that noradrenaline infusion into rats resulted in aggregation of platelets and occlusive platelet thrombi. Haft and Fani [10] reported intravascular aggregation of platelets, similar to that found after noradrenaline infusion, in the hearts of rats subjected to stress. These authors suggested that the catecholamines secreted endogenously during stress are sufficient to cause such platelet aggregation. It is of interest to note that in the Haft and Fani study no fibrin or breakdown of the vessel wall was seen. Nordoy, Gjesdal, Jaeger and Berntsen [21] also observed platelet aggregation induced by both noradrenaline and adrenaline infusion, as well as an increase in the number of circulating platelets, a finding confirmed by the platelet counts of the present study.

It is of interest to consider the role of the glucocorticoids in changes in endothelial permeability and platelet aggregation. The glucocorticoids are reported to protect against the effects of the endogenous inflammatory substances, preventing the leakage of macromolecules, and also the thrombotic complications [27]. Changes such as those reported in this study and the earlier study by Bassett and Cairncross [1] should be inhibited by high circulating steroid levels, and should not be manifest until adaptation of the steroid response to stress has occurred. Measurement of the plasma corticosterone levels over the period of prolonged stress shows a good correlation between the adaptation of the steroid response and the onset of a progressive degeneration of the coronary vascular system. Junctional gaps, deposition of lipid droplets, and changes in platelet number and function really only occur when the plasma steroid level has adapted to its new intermediate level of approximately 45 μg/100 ml plasma by Day 40. When the steroid levels were high (Days 1, 5 and 10) no differences were seen in any of the morphological parameters examined between stressed and control animals.

The possibility that the glucocorticoids may prevent stress induced myocardial pathogenesis by inhibiting the permeability response to inflammatory substances is supported by the work of Garcia Leme and Wilhelm [8] and Davies and Thompson [5]. Garcia Leme and Wilhelm found that the intensity of the permeability response to histamine or serotonin was subject to the influence of adrenal cortical hormones. Corticosterone depressed the vascular permeability response in rats elicited by intracutaneous histamine or serotonin, whereas adrenalectomy enhanced the response. Davies and Thompson reported that the corticosteroids diminished the amount of protein-bound dye passing from the serum into the peritoneal cavity in response to inflammation in the cavity. The glucocorticoids may act not only to inhibit the response to the inflammatory substances but also to stabilize the mast cell membrane and thus prevent the release of inflammatory agents. The production of vascular changes resulting from the release of such substances may result from the destruction of the normal intracellular physiological function of mast cells as proposed by Jaques [14]. Stress induced agents such as the catecholamines result in the release of large amounts of histamine etc. from the mast cell and so induce an anaphylactic response.

From the present study and from the study of Bassett and Cairncross [1] it would appear that prolonged exposure to stress results in pathological changes in the myocardium. At least in part, these changes can be associated with changes in vascular endothelial permeability and platelet aggregation. The role of the glucocorticoids would seem to be to protect against such pathological changes since such changes occur only when adaptation of the steroid response has taken place.

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