Psychological stress activates phosphorylase in the heart of the conscious pig without increasing heart rate and blood pressure
(carbid death/brain regulation/cardiac arrhythmias/cardiac metabolism)

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Communicated by Donald B. Lindsley, April 11, 1983

ABSTRACT The present study uses a technique that enables the collection of multiple freeze-biopsy samples from the myocardium of the conscious pig (i.e., through a thoracic window). This technique enables sequential analysis of the metabolic state of the myocardium during different behavioral conditions. The results demonstrate that with daily adaptations to an unfamiliar environment (i.e., stress reduction), the phosphorylase activation ratio (phosphorylase a/total phosphorylase) in the quiescent pig declines steadily from approximately 80% to 30% (r = −0.91, P < 0.01). This decline occurs with both the mean resting heart rate and left ventricular blood pressure remaining constant. The decline is seen within individual subjects during the whole adaptation sequence as well as between subjects whose samples were taken either early or late in the adaptation series. The dissociation of hemodynamic functional and metabolic activation in the unadapted, psychologically stressed pig may be associated with the occurrence of increased vulnerability of the ischemic heart to ventricular fibrillation, a phenomenon previously observed under the same behavioral conditions.

Psychosocial risk factors in human populations have been identified and appear to be associated with high rates of sudden cardiac death [1-14]. Operationally defined psychological stresses have been shown in conscious animal models to increase the vulnerability of the heart to arrhythmogenesis (15-20). Our laboratory [17, 21-27] has delineated a neurophysiological system that supports Cannon’s (28) early concept of a “cerebral defense system.” Our conceptual model, (22) developed from animal neurophysiological research, shows how a psychologically stressful stimulus in the environment is represented in the cerebrum and translated into vulnerability to arrhythmias in the ischemic heart.

The question addressed in the present study is: how does the psycho-neuro-physiological input to the heart evoke the increased vulnerability to arrhythmogenesis? We have chosen to examine the effects of operationally defined psychological stress on phosphorylase activation in the myocardium. This enzymatic activation is a distal step in an “enzyme cascade” initiated by β-adrenergic stimulation. β-Adrenergic stimulation modulates both cardiac inotropy and metabolism (29). Because phosphorylase activation is a distal step, it is in a position to be sensitive to cross-modulation by other receptor-effector mechanisms in the same cell, such as by α receptors, which increase calcium uptake and, by this mechanism, activate phosphorylase (30). Our present results show in the pig that the psychological stress produced by an unfamiliar environment increases the resting myocardial phosphorylase activation ratio without a concomitant change in either resting heart rate or resting blood pressure. Thus, in a behaviorally controlled condition of psychological stress that is associated with increased cardiac vulnerability, a dissociation is observed between a critical enzyme controlling metabolic energy supply and the actual energy utilization at that moment.

METHODS

Subjects. Prepubertal Hampshire pigs were chosen as the subjects because: (i) pigs manifest a significant degree of sudden cardiac death (31), (ii) the coronary circulation (32) and neural innervation (33) of the pig heart are better homologs of those of the human than are those of the carnivore, and (iii) livestock have a uniform psychological background, in contrast to domesticated carnivores, each of which has been either a family pet or a stray. The strain used by our laboratory is the pure-bred Hampshire pig, produced by the local Huntsville Prison. After 6-9 days of daily experience in the laboratory, these animals will show behavioral and autonomic adaptation; after adaptation, coronary artery occlusion will fail to elicit ventricular fibrillation (in 100% of the cases), whereas before adaptation, this lethal arrhythmia always occurs (17).

Surgical Preparation (Thoracic Window Procedure). The animals were anesthetized with sodium pentobarbital and diethyl ether. A left thoracotomy was made and half of the fourth rib was removed, beginning at the sternum. A prefabricated epoxy window with a silicone rubber plug in its lumen was then inserted in place of the rib and secured to the third and fifth ribs with stainless steel suture wire, as shown in Fig. 1A. The incision was closed tightly with a running stitch. After 3-10 days of postoperative recovery, the animal was brought to the laboratory for the first time, the stitches were removed (after local infiltration of the tissue with lidocaine), and the silicone rubber plug was extracted. After 3 days, sufficient granular tissue forms to seal the edges of the window against the myocardium and prevent air from entering the thoracic cavity. The window was approximately 4 cm wide (rostrocaudal) and 6 cm long (dorsal) and exposed the left myocardium from near its apex to the lower part of the aortae. The granular tissue that covers the myocardial surface can be removed by gentle aspiration. After 5-6 days, a nonremovable vascular network develops that partially obfuscates the view of the epicardial arteries. For longer postoperative recovery periods, the pericardial membrane is left closed during surgery and then opened later, when the rubber plug is first removed.

Cryobiopsy. Fig. 1B illustrates a cryoprobe that has been inserted through a window into the myocardium. The cryogenic biopsy technique is similar to that previously described by Skinner et al. (26) for the in viva inactivation of cerebral tissue.

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In the present case, a 1 mm x 2 mm diameter, 14 mm length, oval cryogenic needle is constructed from untempered, 26 gauge, stainless steel tubing. Because the small polyethylene coolant lines remain flexible, even at low temperatures, they will not create a significant mechanical load and dislodge the cryoprobe after it has been inserted into the beating heart. The warm (37°C) cryoprobe is first inserted into the exposed myocardium through a slit in the epicardial membrane. Cold methanol (−75°C) is circulated through the probe (C), after precooling the delivery line by flow through a shunt line (S). This technique enables the surrounding tissue to be frozen at a very rapid rate (initial rate, 600°C/sec) while heat-sinked by the tissue (26). One to 2 sec after the initiation of freezing, the cryoprobe and adherent tissue are jerked loose from the myocardium, by grasping the probe with a hemostat. Warm methanol is then circulated through the probe to melt the interface between the frozen tissue and the cryoprobe. The frozen tube of tissue is then quickly slipped off of the probe, dropped into liquid nitrogen, and stored. One second of freezing-removing results in a sample whose wall thickness is around 0.5 mm and net weight is around 50 mg. The small hole that remains in the heart muscle after the tissue sample is removed is usually plugged with coagulated blood that results from applying pressure at the epicardial orifice. Occasionally, a Gelfoam strip must be inserted into the hole to stop the bleeding.

All samples in this study were taken from the left ventricle. The field was at least 1 cm lateral to the left anterior descending coronary artery and 1 cm below the circumflex artery. All samples were separated from one another by approximately 1 cm along the epicardial surface.

Behavioral Protocol. Because the chronic psychological state of the animal is the main variable under experimental control, great care and consistency in handling are required. The details are outlined as follows. The pig was brought from the prison facility and allowed 3 days of adaptation to its home cage prior to surgery. After recovery from surgery, the animal was brought to the laboratory, with its legs restrained by adhesive tape. The cryobiopsies were then performed at 5, 30, or 45 min after the exposure of the myocardial surface. After 2 or 3 samples had been taken, during a single daily exposure of the myocardium, the silicone rubber plug was replaced and the animal was returned to its home cage. During each of the subsequent days, the animal was brought back to the laboratory. When cardiac biopsies were not to be performed, the animal was simply attached to the electrophysiological recording leads (electrocardiogram and electroencephalogram). When samples were to be taken, the myocardium was exposed, as described above. In such instances, after the first few experimental days, lidocaine infiltration was not necessary, because the pigs showed only a minimal behavioral reaction to the reexposure of the myocardial field. Each daily observation period required 1–2 hr to complete.

Electrophysiological and Hemodynamic Measurements. Standard limb-lead electrodes were permanently sutured through the skin of the animal's forelegs and left hind leg. Electroencephalogram electrodes were sutured into the scalp. Left ventricular pressure was measured by inserting a 13-gauge needle through the ventricular wall or through one of the holes made by a rapid-freeze biopsy. This method was chosen to eliminate the use of heparin, which is normally required to maintain patency of indwelling catheters. The 13-gauge needle was attached to a calibrated Statham strain gauge transducer. All electrophysiological activities were recorded on a Beckman type R polygraph.

Phosphorylase Assay. Phosphorylase activities were measured by the modification of the linked-enzyme spectrophotometric assay used by Entman et al. (34). Frozen tissue biopsy samples (50–90 mg each) were homogenized in 1.3 ml of 125 mM 2(N-morpholino)ethanesulfonic acid (Mes), pH 6.8/10 mM MgCl2/0.02 mM glucose 1,6-bisphosphate/1 mM EGTA [ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid] at 4°C. Once the tissue was homogenized and placed in a cuvette, the other components of the assay mixture were added to have final concentrations of: 15 mM PO4, 125 mM Mes at pH 6.8, 10 mM MgCl2, 0.02 mM glucose 1,6-bisphosphate, 10 mM NaN3, 1% glycogen, 9.4 units of phosphoglucomutase, 9.4 units of glucose-6-phosphate dehydrogenase, 1 mM EGTA, and 0.5 M NADP. Phosphorylase a activities were measured at 37°C by the linear rate of change in absorbance at 340 nm. Total phosphorylase was then assessed by adding 5 mM 5'-AMP to the cuvette and measuring the absorbance change at 340 nm. This coupled enzyme system has been shown to be linear over the activity and protein ranges measured. There was no evidence of activation or deactivation of phosphorylase a over the course of the reaction under these conditions. We have performed similar assessments of skeletal muscle (35).

RESULTS

Fig. 2 shows a record from a typical animal during a cryobiopsy. The animal reacted with a slight head movement (electroencephalogram artifact) when the cryoprobe was implanted. During the biopsy (freeze extraction), the animal remained quiescent. A whole body movement occurred later, in response to the reinsertion of the blood pressure monitoring needle (movement). In this particular animal, single premature ventricular beats occurred when the cryoprobe was inserted, when the freezing began, and when the tissue was extracted. Single premature beats occurred in most animals; multiple arrhythmias never occurred. The composite results of 32 cryobiopsies, performed on four animals, during adaptation to the unfamiliar sur-
roundings of the laboratory, are shown in Table 1. Each sample was taken after the animal had remained in a quiescent state for at least 5 min. The percent activation of phosphorylase (%P) is shown as the ratio of phosphorylase a to total phosphorylase in the sample. The missing data points, indicated by dashes, are the result of failures to tear the frozen tissue away from the muscle. Note that the resting mean heart rate and blood pressure do not change according to the days of adaptation or %P measured in the biopsy samples. Resting mean heart rate of animals was correlated with their body weight (correlation coefficient, \( r = -0.93 \)).

The weight of each of the samples varied because of the different freeze/extract times around the intended 1-sec interval. The sizes of the samples were not correlated with their %P measurements. The specific site in the myocardium from which the sample was taken also was not correlated with the magnitude of the phosphorylase activation ratio.

Fig. 3 shows the effects of time after exposure of the myocardium on the measurement of phosphorylase activation. It

Table 1. Percent activation of resting phosphorylase, resting heart rate, and resting left ventricular systolic pressure in the pig during psychological adaptation to the unfamiliar surroundings of the laboratory

<table>
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<th>Pig no.</th>
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<th>30 min %P</th>
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%P, percent activation of phosphorylase; HR, heart rate in beats per min; BP, blood pressure in mm Hg.

* Each pig received daily adaptation treatments for 11 consecutive days. Cryobiopsies were performed only on the days indicated. Animal 3 did not receive adaptation treatment between days 11 and 21, as indicated by the values in parentheses.

**The cryobiopsies were performed at approximately 5, 30, and 45 min after the somewhat stressful exposure of the myocardial surface. In all cases, the animal was left undisturbed for at least 5 min so that resting heart rate, blood pressure, and %P could be determined. Each heart rate and blood pressure value shows the mean during the 3-min period prior to the related cryobiopsy in which %P was determined.
can be seen that, at 5 min after the somewhat stressful exposure of the myocardium, phosphorylase is relatively activated in both unadapted (U) and adapted (A) animals. When the samples are taken later, at either 30 or 45 min, the activation has declined in the adapted animals, but not in the unadapted ones ($P < 0.02$). This result shows that a recent phasic stress (e.g., exposure of the myocardium) will reactivate phosphorylase in adapted subjects.

Fig. 4 is a plot of the data from all of the animals, as they adapted to the laboratory over a period of 11 days. To control for the phasic stress effect, only values observed 30 min after exposure of the myocardium were plotted. A statistically significant linear regression ($r = -0.91, P < 0.01$) is seen to occur for the composite 30-min data. The same adaptation effect (i.e., regression) is seen within as well as between subjects. The validity of the 30-min data is confirmed by replication measures at 45 min, as seen in Table 1. These results clearly demonstrate the effect of adaptation to the unfamiliar surroundings of the laboratory on the activation of phosphorylase, with heart rate and blood pressure remaining relatively constant.

**DISCUSSION**

Controls. The low variance of the measurements that leads to the large correlation coefficient shown in Fig. 4 is due, in part, to the behavioral control (i.e., allowing 30 min to pass after the exposure of the myocardium to eliminate the effects of recent stress) and to the choice of the phosphorylase ratio as the measure (i.e., the ratio makes the measure independent of sample size). Muscle cells contain a high level of phosphorylase compared to that of other cells in the cryogenic sample; thus, the additional source of random variance attributable to non-muscle tissues is minimized.

Several observations preclude some possible explanations of the regression seen in Fig. 4. The steady decline of phosphorylase activation over days cannot be explained by the number of previous samples taken from a given heart. For example, animal 4 in Table 1 shows approximately the same low degree of phosphorylase activation after adaptation (i.e., 10–11 days) as do the other pigs, but the former subject had only a few previous samples taken, whereas the others had numerous ones. Also, sample size and location have been ruled out as a possible explanation. The result also cannot be explained by a fixed decline in phosphorylase activation over days, because reactivation occurs within each subject after a mild stress (Fig. 3, 5 min). And, finally, because neither heart rate nor blood pressure is correlated with phosphorylase activation (Table 1), a change in cardiac chronotropy or inotropy cannot explain the decline in phosphorylase activation during adaptation.

**Phosphorylase Mechanisms.** The sympathetic nervous system increases the pumping power of the heart and activates the metabolic support system for the increased work load. One mechanism of this increase in metabolic supply occurs through the activation of an enzyme, phosphorylase. Adrenergic initiation of the activation sequence that ultimately activates cardiac phosphorylase begins with the production of adenosine 3',5'-monophosphate (cyclic AMP) after the receptor activation of the underlying adenylyl cyclase (36–38). The mechanism of action of cyclic AMP appears to be via its activation of protein kinase (39). The active form of this latter enzyme catalyzes the adenosine triphosphate-dependent transformation of phosphorylase kinase to its active form. Activated phosphorylase kinase then catalyzes the phosphorylation of phosphorylase $b$, converting it to its active form, phosphorylase $a$. The final activation of phosphorylase then initiates the rapid and intense stimulation of glycogenolysis (40–43).

A dissociation of the hemodynamic and metabolic activation of the heart can occur via several different mechanisms. Blu-koo-Altoey et al. (44) showed that acetylcholine, in concentrations that are shown to have no effect on inotropy, could inhibit adrenergic activation of phosphorylase. Others have shown that such cholinergic agents will prevent the adrenergic stimulation of cyclic AMP formation (45–47). This cholinergic effect in the heart (49, 48) is associated with an inhibition of the effects of guanine nucleotides. These nucleotides normally alter the affinity of $B$ receptors to their agonists and are essential for the hormonal activation of adenylyl cyclase (48). The phosphodiesterase inhibitor papaverine can increase cyclic AMP and activate phosphorylase without changing cardiac inotropy (50). Thus, both increases and decreases of phosphorylase activation can occur with cardiac inotropy remaining constant.

In the heart made vulnerable to arrhythmias by ischemia, both cyclic AMP increase and phosphorylase activation occur; both responses can be blocked, in part, by competitive inhibition of the $B$ receptor (29, 51). In some experiments with anoxia, however, $B$-receptor blockade prevents the rise in cyclic AMP and protein kinase activation, but phosphorylase $a$ formation still occurs (52). The mechanism by which phosphorylase is activated independently of cyclic AMP and protein kinase could involve an increase in Ca$^{2+}$ flux (52) or an alkaline shift in pH (33), because either one of these alterations produced in vitro will directly augment phosphorylase kinase activity and increase the conversion of phosphorylase $b$ to phosphorylase $a$.

**Dual Autonomic Regulation and Ventricular Ectopy.** A possible mechanism to explain the present results is that a shift in the ratio of muscarinic and $B$-receptor activity occurs during adaptation, a situation that keeps heart rate constant but shifts the phosphorylase activation ratio. That this type of dual autonomic regulation of the heart can occur is demonstrated in a study by Baust and Bonnert (54). They found that although mean heart rate remains constant during sleep, high sympathetic tone and high vagal activity occur during slow wave sleep, whereas only a persistent vagal tone occurs during rapid eye movement sleep. Sleep studies performed in our laboratory have shown, in the infarcted pig heart, that arrhythmia rate is markedly reduced during rapid eye movement sleep relative to the immediately adjacent slow wave sleep interval (55). These two studies together suggest that high sympathetic tone, with
chronotropy suppressed by vagal activity, increases cardiac vulnerability to arrhythmogenesis. Extended to the present study, this interpretation would suggest that high sympathetic and high vagal tone occur together during the resting state in the unadapted pig because its heart is more vulnerable to arrhythmogenesis (17) than that of the adapted animal. This same pattern of dual autonomic regulation could also explain the relatively greater activation of phosphorylase in the resting heart of the unadapted pig via the known mechanisms of metabolic and hemodynamic control.

This research was supported by grants from the National Institutes of Health: HL 25425 and HL 17907 to J. E. S. and HL 13870 and HL 23161 to M. E. S. D. B. was a Special Fellow with the Department of Pediatric Cardiology, Texas Children's Hospital (HL 07192), who worked on this research in the Section of Neurophysiology.