REDOX STATE OF NICOTINAMIDE-ADENINE DINUCLEOTIDE
IN THE INNER AND OUTER LAYERS OF CANINE LEFT
VENTRICLE, AND EFFECTS OF CORONARY
DILATORS ON THE REDOX STATE

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Abstract - The effects of nitroglycerin, dipyridamole, papaverine and anoxia on the
cellular redox state were investigated in the inner and outer layers of the left ventricu-
lar wall, in open-chest dogs anesthetized with morphine and pentobarbital. As
an index of the cellular redox state, the NAD+/NADH ratio was employed. The
NAD+/NADH ratio was calculated by direct measurements of NAD+ and NADH
in the tissue. In the control dog, the NAD+/NADH ratio in the inner layers was
usually lower than that in the outer; a transmural gradient of the NAD+/NADH
ratio across the left ventricle was detected. Anoxia was produced by discontinua-
tion of artificial respiration for 3 min. In the anoxic dog, the NAD+/NADH ratios
in both the layers were markedly lower than those obtained in the control dog. The
transmural gradient of the NAD+/NADH ratio was not modified by the injection of
either dipyridamole (250 μg/kg, i.v.) or papaverine (2 mg/kg, i.v.), but it was in-
creased by the production of anoxia, while it was abolished by the injection of nitro-
glycerin (20 μg/kg, i.v.). The possible mechanisms of the beneficial action of nitro-
glycerin against angina pectoris are discussed with special reference to the myocardial
redox state.

It has been demonstrated from histological findings that endocardial (or inner) layers
of the left ventricle are more vulnerable to ischemia than epicardial (or outer) layers (1, 2).
Blood flow studies have indicated that regional blood flow in the inner layers is reduced
more than that in the outer layers when the total coronary blood flow is decreased (3, 4).
Some investigators have shown that tissue oxygen tension (pO2) of the inner layers is less
than that of the outer even under normal conditions (5–8). Thus, there is a view that
the inner layers are slightly ischemic when compared to the outer, even under the normal
state of coronary blood flow (9–12). This view corresponds with the findings that the
level of glycogen, activity of phosphorylase, and lactate/pyruvate ratio of the inner layers
are higher than those of the outer in the normal heart (11, 13).

Although the precise mechanisms of the action of nitrites to improve anginal attack
are still obscure, Winbury et al. (14) proposed the hypothesis that nitrites produce redis-
tribution of blood flow from the outer layers to the inner in the ventricular myocardium,
and that the redistribution is responsible for their beneficial effect against anginal attack. This hypothesis is partially supported by the fact that tissue pO₂ in the inner layers is increased by the injection of nitroglycerin (6). This finding, if valid, would suggest that the redox state of the inner layers changes to a more oxidized one with the administration of nitrates.

The present study was undertaken to examine whether there is a transmural gradient in the redox state of the left ventricle even under normal conditions, and to investigate the effect of coronary dilators on the redox state in the inner and outer layers. As an index of the cellular redox state, the ratio of the oxidized form of nicotinamide-adenine dinucleotide (NAD⁺) to its reduced form (NADH) was employed in this study.

MATERIALS AND METHODS

Forty-eight mongrel dogs of both sexes weighing between 6 and 14 kg, 8.76 ± 0.29 kg (mean ± S.E.), were used. Dogs were anesthetized by a s.c. injection of morphine hydrochloride (10 mg/kg) followed after 30 min by an i.v. injection of sodium pentobarbital (15 mg/kg). After endotracheal intubation, respiration was controlled using a positive pressure respirator. The left side of the thorax was opened to permit free access to the left ventricle. The left ventricle was rapidly removed with scissors from the beating heart, and immediately frozen in liquid nitrogen. In experiments with anoxia, the left ventricle was removed 3 min after discontinuation of the respirator. In experiments with drugs, the left ventricle was removed 10 min after start of the drug injection. The drugs used were nitroglycerin, dipyridamole, and papaverine hydrochloride and were injected i.v. over a period of 30 sec. (Nitroglycerin is an effective antianginal drug, but this effect lasts only for 15-30 min (15). Therefore, it was decided that the ventricle should be removed within 15 min after the injection of nitroglycerin in order to evaluate the antianginal effect of nitroglycerin. Our previous paper (16) has shown that nitroglycerin and papaverine produce marked circulatory changes immediately after injections of the drugs, and the changes last for a period of about 10 min. Accordingly, the left ventricle was removed 10 min after injections of drugs in the present study.

The frozen left ventricular myocardium was then placed on a block of dry ice, and was divided into two; the inner and outer halves (or layers). Each of the two layers was powdered in a mortar previously cooled with dry ice. Three hundred milligrams of frozen tissue powder were taken twice from each of the two myocardial layers for the measurements of NAD⁺ and NADH respectively. The extractions of NAD⁺ and NADH were carried out by the method described by Ciotti and Kaplan (17). NAD⁺ was separated from NADP⁺, and NADH from NADPH by the method of Lowry et al. (18). These methods enable extraction of either NAD⁺ or NADH from the tissue in the form of NAD⁺. The amount of NAD⁺ was determined by the strong alkali method of Kaplan et al. (19) using the Farrand spectrofluorometer (activation wavelength = 360 nm, emission wave length = 455 nm). The recoveries of NAD⁺ and NADH were 88.0% and 100.8% respectively. Details of this procedure are described below.
TABLE 1. Procedures for extraction and determination of NAD$^+$ and NADH in the myocardium

| Procedure                                                                 | Solution A                                                                 | Solution B                                                                 | Solution C                                                                 |
|---------------------------------------------------------------------------|---------------------------------------------------------------------------|---------------------------------------------------------------------------|---------------------------------------------------------------------------|
| Tissue powder (frozen)                                                    |                                                                            |                                                                            |                                                                            |
| 1) homogenize with 5% TCA                                                 |                                                                            |                                                                            |                                                                            |
| 2) centrifuge                                                             |                                                                            |                                                                            |                                                                            |
| 3) wash with ether                                                        |                                                                            |                                                                            |                                                                            |
| A) NAD$^+$ + NADP$^+$                                                     | Tris buffer (pH 8.2)                                                      |                                                                            |                                                                            |
| G-6-P                                                                     |                                                                            |                                                                            |                                                                            |
| G-6-P dehydrogenase                                                       |                                                                            |                                                                            |                                                                            |
| A') NADH - NADPH                                                         |                                                                            |                                                                            |                                                                            |
| neutralize with 1N HCl                                                     |                                                                            |                                                                            |                                                                            |
| Tris buffer (pH 8.2)                                                      |                                                                            |                                                                            |                                                                            |
| acetaldehyde                                                              |                                                                            |                                                                            |                                                                            |
| alcohol dehydrogenase                                                     |                                                                            |                                                                            |                                                                            |
| EDTA                                                                      |                                                                            |                                                                            |                                                                            |
| B) NAD$^+$ + NADPH                                                        |                                                                            |                                                                            |                                                                            |
| acid                                                                      |                                                                            |                                                                            |                                                                            |
| C) NAD$^+$                                                                |                                                                            |                                                                            |                                                                            |
| strong alkali (boil)                                                      |                                                                            |                                                                            |                                                                            |
| Fluorescent product                                                       |                                                                            |                                                                            |                                                                            |
| Activation wave length = 360 nm                                           |                                                                            |                                                                            |                                                                            |
| Emission wave length = 455 nm                                             |                                                                            |                                                                            |                                                                            |

Extraction and determination of tissue NAD$^+$ (Table I)

The frozen tissue powder (300 mg) was transferred to a glass homogenizer containing 6.0 ml of ice-cold 5% trichloroacetic acid, and was homogenized. The homogenate was then centrifuged at 6,500 r.p.m. for 15 min. The supernatant solution of the homogenate was washed with ether three times in order to remove the trichloroacetic acid. The solution thus obtained is Solution A. To 1.0 ml of Solution A were added 4.0 ml of 0.05 M Tris buffer (pH 8.2), 0.2 ml of glucose-6-phosphate (10 mg/ml) and 0.1 ml of glucose-6-phosphate dehydrogenase (12.5 units/ml) to give Solution B. The reaction was allowed to proceed for 10 min at a room temperature of about 20°C. (In in vitro experiments, it was confirmed that 300 μg of NADP$^+$ was reduced by 100% in 5 min in the presence of 32 units of glucose-6-phosphate dehydrogenase. In other experiments, it was confirmed that 12.5 units/ml of glucose-6-phosphate dehydrogenase was enough to reduce NADP$^+$ contained in the myocardium.) To 1.0 ml of Solution B was added 0.1 ml of 1.3 N HCl, and to the HCl-mixed Solution B was added 1.0 ml of water to give Solution C. Then, to the Solution C was added 2.0 ml of 8.75 N NaOH. The NaOH-mixed
Solution C was placed in a boiling-water bath for 5 min, and then cooled. Finally, the fluorescence of the cooled NaOH-mixed Solution C was measured in terms of NAD'.

**Extraction and determination of tissue NADH (Table 1)**

The frozen tissue powder (300 mg) was transferred to a glass homogenizer containing 6.0 ml of boiled 0.1 M Na₂CO₃ solution, and the homogenizer was placed in a boiling-water bath for 30 sec. The tissue powder was then homogenized. The homogenate thus obtained was placed again in the boiling-water bath for 30 sec, and was centrifuged at 8,500 r.p.m. for 15 min. Thus the supernatant solution of the homogenate was obtained (solution A'). The Solution A' (1.0 ml) was neutralized with 0.1 ml of 1N HCl. To the Solution A' neutralized with HCl were added 3.9 ml of 0.05 M Tris buffer (pH 8.2), 0.1 ml of alcohol dehydrogenase (186 units/ml), 0.1 ml of 1% acetaldehyde, and 0.1 ml of 45 mM EDTA, to give Solution B'. The reaction was allowed to proceed for 10 min at a room temperature of about 20°C. (In *in vitro* experiments, it was confirmed that 400 μg of NADH was oxidized by 100 units in 5 min in the presence of 153.5 units of alcohol dehydrogenase. In other experiments, it was confirmed that 186 units/ml of alcohol dehydrogenase was enough to oxidize NADH contained in the myocardium). To 1.0 ml of the Solution B' were added 0.1 ml of 1.3 N HCl and 1.0 ml of water to give Solution C'. Then, to the Solution C' was added 2.0 ml of 8.75 N NaOH. The NaOH-mixed Solution C' was placed in a boiling-water bath for 5 min and then cooled. Finally, the fluorescence of the cooled NaOH-mixed Solution C' was measured in terms of NAD'.

**RESULTS**

The mean levels of NAD' and NADH, and the mean NAD'/NADH ratio in the inner and outer layers of the left ventricular myocardium are summarized in Table 2.

**Control experiments**

In control experiments using 15 normal dogs, no significant differences between the inner and outer layers were detected in the mean level of NAD' and that of NADH. There was, however, a significant difference in the NAD'/NADH ratio between both the layers: the mean NAD'/NADH ratio in the inner layers (1.700±0.077) was significantly lower (P<0.05) than that in the outer (1.914±0.067). This evidence indicates that there is a transmural gradient of the NAD'/NADH ratio across the normal left ventricular myocardium. If the NAD'/NADH ratio is a suitable index of the cellular redox state, it can be postulated that there is a transmural gradient of the redox state across the normal myocardium. The above-mentioned transmural gradient of the NAD'/NADH ratio was observed in 11 out of 15 normal dogs tested. In the remaining 4 dogs, however, a reversed transmural gradient of the NAD'/NADH ratio was present. The discrepancy has yet to be elucidated. However, it is possible that the redox state of the inner layers is not always lower than that of the outer under normal conditions.

**Experiments with anoxia**

In experiments with anoxia using 7 dogs, the mean level of NADH in the inner layers
(542.51 ± 18.55 nmol/g wet weight) and that in the outer (469.61 ± 15.85) were significantly higher than those in the respective layers of the control dog (inner layers: 380.61 ± 13.24; outer layers: 361.64 ± 13.40). Since the mean level of NAD⁺ in each of the two layers of the anoxic dog was not significantly different from that in the respective layers of the control dog, the NAD⁺/NADH ratio in each of both the layers of the anoxic dog heart (inner layers: 1.195 ± 0.038; outer layers: 1.549 ± 0.062) was significantly lower than that in the respective layers of the control dog heart (inner layers: 1.700 ± 0.077; outer layers: 1.914 ± 0.067).

The significant differences between the two layers in the level of NAD⁺ (inner layers: 645.06 ± 16.48 nmol/g wet weight; outer layers: 721.72 ± 15.76) and that of NADH (inner layers: 542.51 ± 18.55; outer layers: 469.61 ± 15.85), and in the NAD⁺/NADH ratio (inner layers: 1.195 ± 0.038; outer layers: 1.549 ± 0.062) were detected in the anoxic dog; in the inner layers, the mean level of NAD⁺ and NAD⁺/NADH ratio was lower, and the mean level of NADH was higher than those in the outer layers. In all the anoxic dogs, the transmural gradient of the NAD⁺/NADH ratio across the left ventricular myocardium was detected. It is noteworthy that the transmural gradient of the NAD⁺/NADH ratio in the anoxic dog was more prominent than that in the control dog.
Experiments with nitroglycerin

In 9 normal dogs, the effect of nitroglycerin (20 μg/kg, i.v.) on the NAD⁺/NADH ratio in the inner and outer layers of the left ventricle was studied. There were no significant differences in the mean levels of NAD⁺ and NADH, and mean NAD⁺/NADH ratio between the two layers in the nitroglycerin-injected dog. There were also no differences in the levels of NAD⁺ and NADH in each of the layers between the control and nitroglycerin-injected dogs. It should be noted that the NAD⁺/NADH ratio in the inner layers (1.701 ± 0.094) was not significantly different from that in the outer (1.731 ± 0.097). Since in the control dog the NAD⁺/NADH ratio in the inner layers was significantly lower than that in the outer, it was concluded that nitroglycerin abolished the transmural gradient of the NAD⁺/NADH ratio across the myocardium which existed in the control dog heart. The NAD⁺/NADH ratio in the outer layers in the nitroglycerin-injected dog was lower than that in the control, but the difference was not statistically significant.

Experiments with dipyridamole

In 8 normal dogs, the effect of dipyridamole (250 μg/kg, i.v.) on the NAD⁺/NADH ratio in the left ventricle was studied. There were no significant differences in the mean levels of NAD⁺ and NADH between the two layers. The mean levels of NAD⁺ and NADH, and mean NAD⁺/NADH ratio in each of the two layers of the dipyridamole-injected dog were not significantly different from those in the respective layers of the control dog. The NAD⁺/NADH ratio in the inner layers (1.572 ± 0.058), however, was significantly lower (P<0.05) than that in the outer (1.849 ± 0.098) in the dipyridamole-injected dog, as was observed in the control dog. This finding leads to the suggestion that dipyridamole does not modify the redox state of the normal ventricular myocardium. The NAD⁺/NADH ratio in the inner layers in the dipyridamole-injected dog was lower than that in the control dog, but this difference was not statistically significant.

Experiments with papaverine

In 9 normal dogs, the effect of papaverine (2 mg/kg, i.v.) on the NAD⁺/NADH ratio in the left ventricle was studied. There were no significant differences in the mean levels of NAD⁺ and NADH between the two layers. The mean levels of NAD⁺ and NADH, and mean NAD⁺/NADH ratio in each of the two layers of the papaverine-injected dog were not significantly different from those in the respective layers of the control dog. The NAD⁺/NADH ratio in the inner layers (1.710 ± 0.063), however, was significantly lower (P<0.05) than that in the outer (1.940 ± 0.075) in the papaverine-injected dog, as observed in the control and dipyridamole-injected dogs. This result suggests that papaverine, like dipyridamole, does not modify the normal myocardial redox state in the left ventricle.

DISCUSSION

The redox state of pyridine nucleotides has been considered to be one of the most sensitive indicators of the functional state of cellular oxidation (20). The ratio of the concentrations of free NAD⁺ and NADH at the site of consideration is of special impor-
CORONARY DILATORS ON MYOCARDIAL REDOX STATE

tance, because it reflects the metabolic behaviour of oxidizable and reducible substrates, and there are methods which make it possible to calculate the free NAD$^+$/NADH ratio in the cytoplasm, mitochondrial cristae, and mitochondrial matrix using suitable NAD-linked substrates (21). These methods, however, are valid only when the equilibrium constants of the NAD-linked dehydrogenase systems do not change. In the present experiments in which either anoxia was produced or coronary dilators were injected, the equilibrium constants would be expected to change. For this reason, the NAD$^+$/NADH ratio was calculated by direct measurements of NAD$^+$ and NADH.

Although there is a dispute about the meaning of the NAD$^+$/NADH ratio calculated by direct measurements of NAD$^+$ and NADH (21), the NAD$^+$/NADH ratio thus calculated did decrease greatly under the condition of anoxia. This result is feasible (because the NAD$^+$/NADH ratio is decreased by the production of anoxia), and corresponds with the results of Michal et al. (22) on changes in myocardial pyridine nucleotides during anoxia. Such being the case the NAD$^+$/NADH ratio calculated by direct measurements of NAD$^+$ and NADH was employed as an index of the cellular oxidation in the present study.

The present experiments demonstrated that the NAD$^+$/NADH ratio in the inner layers (1.700±0.077) was significantly lower than that in the outer (1.914±0.067) under the normal conditions. This result corresponds with the fact that tissue pO$_2$ in the inner layers is lower than that in the outer (5-8). Why there is a transmural gradient of the NAD$^+$/NADH ratio across the left ventricular wall has yet to be clarified, but there is the possibility that this is due to the under-perfusion of the inner layers, as suggested by Kirk et al. (10), or to a greater consumption of oxygen by the inner layers, which contract more vigorously (23). A significant difference in the NAD$^+$/NADH ratio between the inner and outer layers of the left ventricular wall was detected also in experiments with anoxia (inner: 1.195; outer: 1.549), dipyridamole (inner: 1.572; outer: 1.849), or papaverine (inner: 1.710; outer: 1.940), but not in the experiment with nitroglycerin (inner: 1.701; outer: 1.731). The most prominent degree of the transmural gradient of the NAD$^+$/NADH ratio was observed in the anoxic dog. It is of interest that only nitroglycerin abolished the transmural gradient of the NAD$^+$/NADH ratio across the myocardium. It has been well established that dipyridamole and papaverine are excellent coronary dilators (15). Nitroglycerin, however, is not so excellent in this regard, and in addition, nitroglycerin does not dilate the coronary vessels in patients with coronary sclerosis and angina pectoris (24). Cowan et al. (25) observed that nitroglycerin administered sublingually increased myocardial blood flow in both normal subjects and patients with coronary heart disease. However, this increase in myocardial blood flow was observed to take place only during the early post-administrative stage. Therefore, mechanisms other than coronary dilatation appear to be responsible for the antianginal action of nitroglycerin. According to Winbury et al. (14), nitrites produce a redistribution of blood from the outer layers to the inner. If this hypothesis is correct, nitroglycerin would increase regional blood flow in the inner layers at the expense of regional blood flow in the outer.
and as a result the NAD⁺/NADH ratio in the inner layers would increase to produce the myocardial NAD⁺/NADH ratio or redox state in the inner and outer layers homogeneous. The results obtained in the present study suggest that nitroglycerin makes the transmural difference in the redox state of the left ventricular wall homogeneous. Whether or not the change in myocardial redox state produced by nitroglycerin is due to redistribution of myocardial blood flow remains the subject of future study.

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