Virchows Arch. A Path. Anat. and Histol. 388, 69–76 (1980)

Virchows Archiv A

Impaired Sarcolemmal Membrane Permeability in Reperfused Ischemic Myocardium

Ultrastructural Tracer Study

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Summary. Sarcolemmal membrane permeability to intravenously injected horseradish peroxidase HRP (MW=40,000) was examined in 8 Wistar rats which had temporary ischaemia produced by left coronary artery ligation. HRP reaction product was identified following 6 min of circulation time by light and electron microscopy. Controls included 4 uninjected animals with coronary ligation, 2 uninjected animals without myocardial ischaemia and 2 injected non operated rats.

In normal myocardium, the tracer permeated endothelial plasmalemmal vesicles, intercellular spaces and intracellular vesicles of the T-tubule system, but never permeated the cytoplasm of myocardium cells.

As early as 15 min after coronary artery ligation followed by 6 min of reperfusion with circulation of the tracer, HRP product could be seen in the cytoplasm of muscle cells randomly distributed in the subendocardial area. The quantity of permeated cells increased when the ischaemic myocardium is reperfused during 10 min before injecting the tracer.

These data indicate that sarcolemmal membrane alteration is an early event in myocardial ischaemic injury and precede the irreversible cellular degenerative changes.

Key Words: Myocardial ischaemia – Membrane permeability – Horseradish peroxydase – Electron microscopy.

Introduction

Restoration of blood flow through various organs following temporary ischaemia results in the exclusion of large areas from the effects of reperfusion (Majno et al. 1967; Chaing et al. 1968; Willms Kretschmer et al. 1969; Summers et al.

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This work was supported by research grant from INSERM (ATP 78-95)

0340-1227/80/0388/0069/S01.60

1971). In the myocardium as well as in other organs such impairment of blood flow has been explained on the basis of hypoxic cellular swelling (Krug et al. 1966; Kloner et al. 1974; Willerson et al. 1977). In a previous study, we stressed that this so-called "no reflow" phenomenon could be detected after less than 30 min of coronary clamping in the rat (Camilleri et al. 1976; Deloche et al. 1977). In this phase, endothelial cell swelling appear to be of minor significance. Using carbon black injection, we showed that the hypoperfused areas were characterized by a collapse of the capillary bed mainly due to interstitial and myocardial cell oedema (Camilleri et al. 1976). These data suggest a selective loss of permeability of the muscle dell in the early phase of acute myocardial ischaemia.

In order to visualize these changes in reperfused ischaemic myocardium, we investigated the sarcolemmal membrane permeability using the fine structural extracellular protein tracer, horseradish peroxidase (HRP).

Material and Methods

Male Wistar rats weighing an average of 250 g were used. Left coronary artery ligation was carried out with the simplified method of Selye et al. (Selye et al. 1960) as modified by Deloche et al. (Camilleri et al. 1976; Deloche et al. 1977). This method allows reperfusion after variable times of ischaemia by traction on the exteriorized portion of the suture.

Eight rats with myocardial ischaemia of 15 or 30 min duration were injected with the tracer either immediately after reestablishment of blood flow (4 rats) or after 10 min of reperfusion (4 rats). 10 mg/100 g body weight of horseradish peroxidase HRP (40,000 daltons) (Sigma Chemical Co, type II) dissolved in 0.5 mg of saline were injected intravenously under ether anesthesia and allowed to circulate for 6 min before the animals were killed by fixation-perfusion. The aorta was catheterized and the catheter attached to a perfusion pump with a constant flow rate of 50 ml/mn. Following injection of heparin and potassium chloride, fixation was performed immediately by perfusing a solution of 2.5% glutaraldehyde in a 0.1 M sodium cacodylate buffer pH 7.4 (final osmolality = 610 mOsmoles/l). Glutaraldehyde perfusion was maintained for 5 minutes while allowing the fixative to escape through the divided vena cava. Two *normal control*, one of which was sham operated, were injected with the tracer.

After perfusion-fixation thin slices of heart were obtained 0.5 to 0.6 cm from the apex. Four randomly selected blocks were taken from each area (anterior, lateral, posterior and septal walls of the left ventricle), i.e., 16 blocks from each animal. All specimens were immersed in the aldehyde fixative for 2 h at room temperature and washed overnight in cacodylate buffer at 4° C (final osmolality = 495 mOsmoles/l). Demonstration of the peroxidase was performed on 20 μm sections. These sections were incubated for 1 h at room temperature in Graham-Karnowsky medium containing 3.3′ diaminobenzidine tetrahydrochloride and H_2O_2 (8). Tissue were post-fixed for 2 h with 1% OsO_4 in phosphate buffer at 4° C, dehydrated in graded alcohols and embedded in Epon 812. One micron unstained sections were prepared for light microscopy. Thin sections were cut with a diamond knife on a Reichert microtome and examined using a Siemens Elmiskop 101 electron microscope either unstained or following staining with lead citrate. – *Control sections* were incubated in medium without hydrogen or without diaminobenzidine.

Uninjected animals (2 normal rats and 4 rats with 15 or 30 min ischaemia with or without reperfusion) were used to evaluate endogenous peroxidase reaction and false positive reactions due to extracellular diffusion of haemoglobin. To exclude artefactual absorption of peroxidase on altered cells, sections of tissue from uninjected normal or ischaemic myocardium were soaked in buffer containing peroxidase and processed for electron microscopy as described for sections from in vivo injected animals.

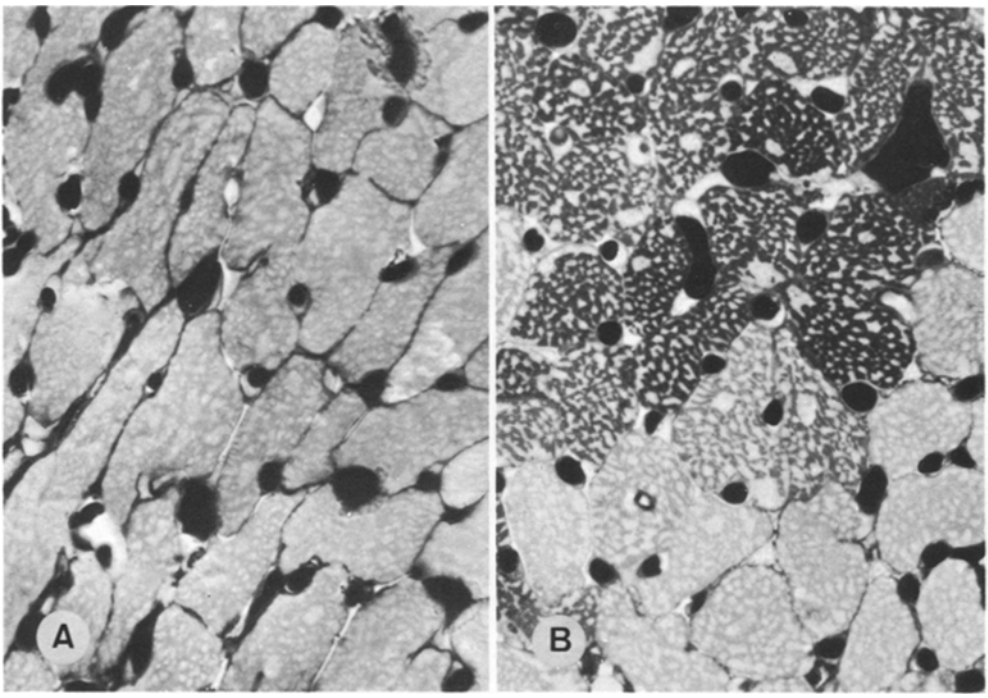

Fig. 1.A Control rat myocardium following 6 min of HRP circulation time. Note regular distribution of reaction product in the interstitium. The myocardial cells are never penetrated by the tracer. One micron unstained section from epon-embedded tissue G=945×. **B** Myocardium after 10 min reperfusion following 30 min ischaemia. HRP reaction product can be seen in the cytoplasm of neighbouring cells. One micron unstained section from epon-embedded tissue G=945×

Results

In control rats killed 6 min after the injection of HRP, the enzymatic tracer was evenly distributed in the interstitium and formed an areolar network closely applied to the myocardial cells on transverse sections of muscle bundles (Fig. 1 A). Upon electron microscopic examination, peroxidase reaction product was seen in pericapillary and intercellular spaces (Fig. 2). It was also seen in endothelial and subsarcolemmal pinocytotic vesicles (Fig. 3). The cytoplasm of muscle cells was never penetrated by the tracer. In control rats which had not been injected with the tracer, no reaction product could be seen in interstitial spaces. In some lysosome-like bodies of the myocardial cells, endogenous peroxidase could be detected.

In reperfused ischaemic myocardium, the reaction product allowed us to visualize the irregularly widened intercellular spaces. The intensity of reaction was different in various areas when compared with the normal pattern. While the interstitium adjacent to perfused capillaries was intensely marked for HRP, the reaction surrounding myocardial cells appeared irregularly distributed. Furthermore, HRP was also seen in the cytoplasm of the myocardial cells (Fig. 1 B).

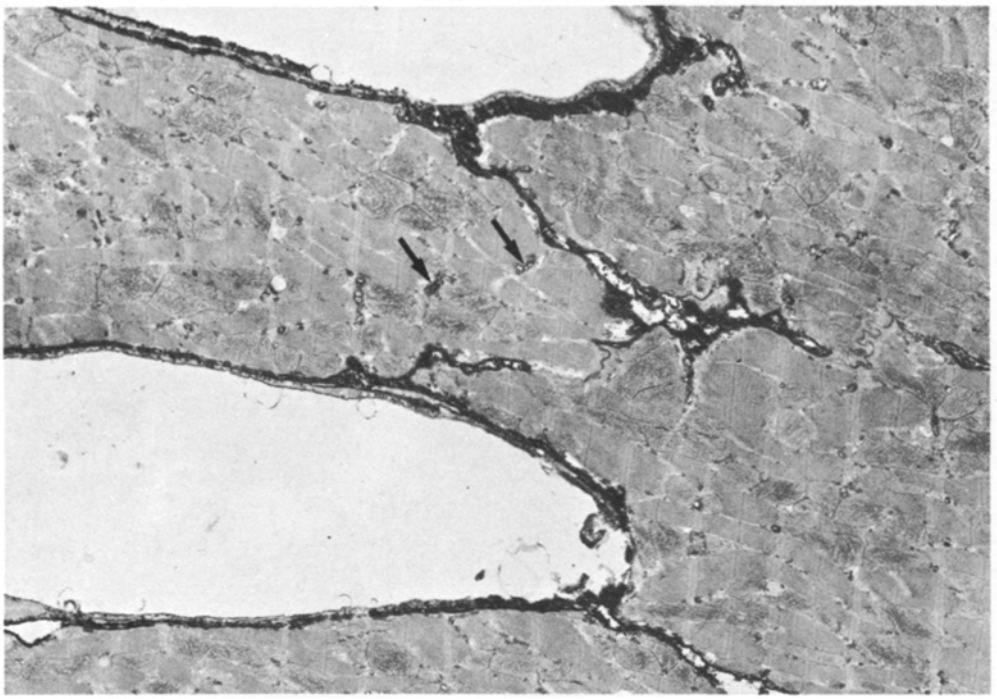

Fig. 2. Control rat myocardium following 6 min of HRP circulation time Pericapillary and interstitial spaces exhibit peroxidase reaction product. The tracer is also seen in the vesicles of the T-tubule system (*arrow*). Thin unstained section, G = 8,000 ×

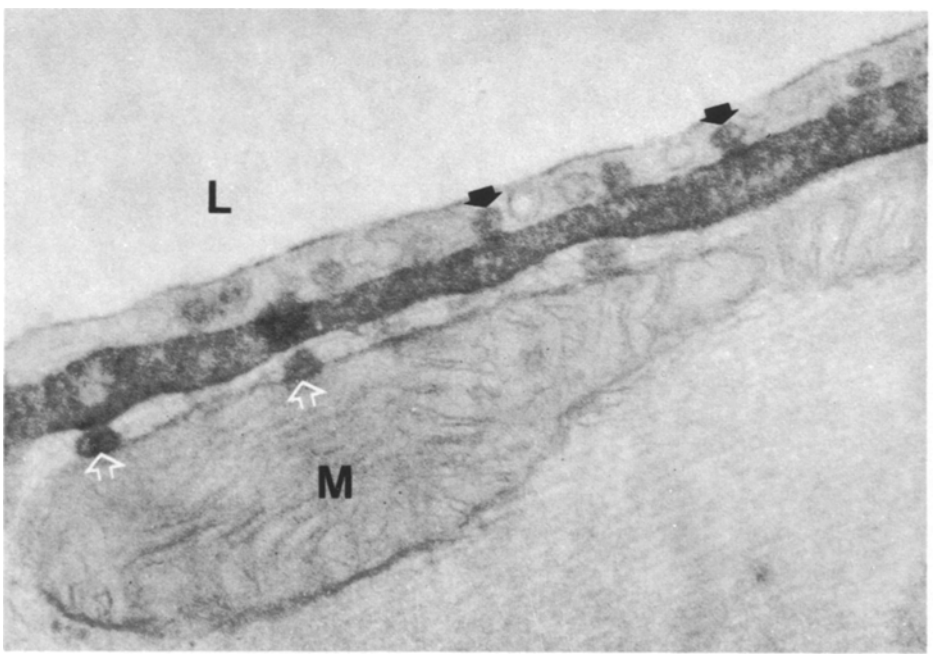

Fig. 3. Control rat myocardium following 6 min of peroxidase circulation time. Note HRP reaction product in endothelial vesicles (*arrows*), interstitial spaces and subsarcolemmal vesicles (*open arrows*). Vesicles on the blood front are not labelled. *M* = mitochondria, *L* = Lumen. Unstained section × 104,000

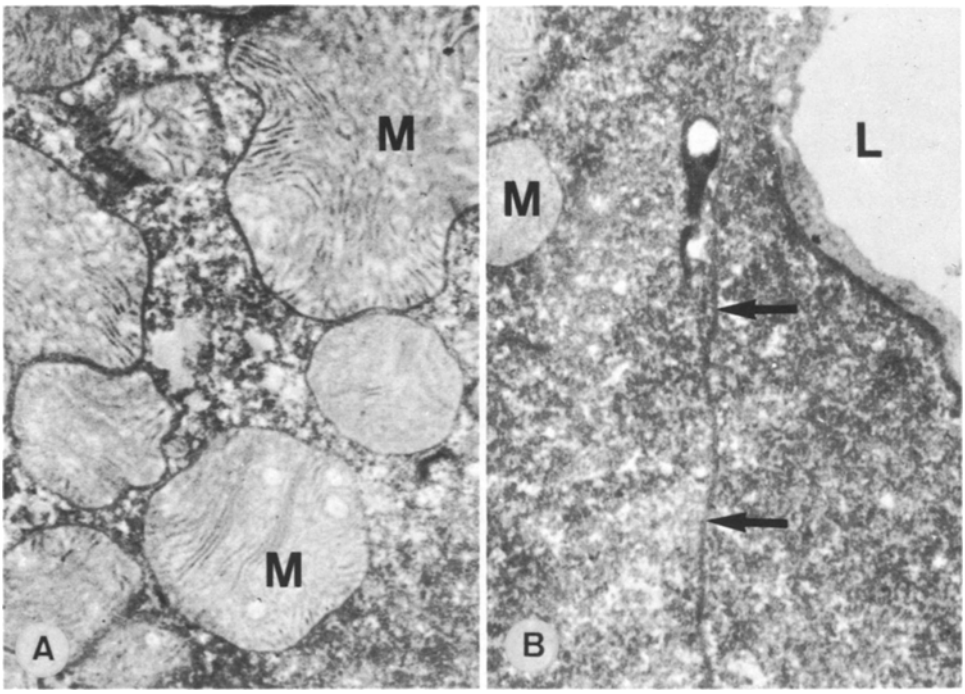

Fig. 4A and B. Cardiac muscle cell from left ventricular myocardium after 10 min reperfusion following 30 min ischaemia. **A** Heavy deposits of HRP reaction product are evident in the cytoplasm. Note the labelling of the external membranes by the tracer. *M* = mitovhondria. Unstained section × 28,000. **B** Dense HRP reaction product can be seen in the interstitial space and in the sarcoplasm while the sarcolemmal membrane (*arrow*) appears to be continuous. *M* = mitochondria, *L* = Lumen. Unstained section × 24,000.

After 15 min of blood flow interruption and 6 min of reperfusion with HRP circulation, the tracer could only be detected in a few isolated subendocardial muscle cells. After 15 min of ischaemia followed by 10 min of reperfusion and 6 min of HRP circulation, reaction product was noted in more numerous myocardial cells randomly distributed throughout the myocardium. After 30 min of ischaemia, the tracer appeared within clumps of neighbouring cells. In myocardium that had been ischaemic for 30 min and reperfused for 10 min before injecting the tracer, muscle cells showing intracellular HRP were twice as numerous. Most of them were located in the subendocardial areas. The density of reaction product was different from cell to cell.

Electron microscopy identified an intracytoplasmic localization for HRP in ischaemic cells (Fig. 4A). In permeated cells the sarcolemmal membrane appeared to be continuous (Fig. 4B). On sections stained with lead citrate, most of these cells showed slight intracellular oedema and glycogen depletion but were otherwise structurally normal. Other cells exhibited heavy reaction product in sarcoplasmic areas and altered myofilaments. The latter aspect was coincident with marked intracellular oedema, dilatation of sarcoplasmic reticulum and mitochondrial alterations such as linear densities and clearing of matrix.

Discussion

Several studies have demonstrated that reperfusion following a period of myocardial ischaemia, results during the first minutes in a rapid uptake of sodium, water and calcium (Shen and Jennings 1972; Leaf 1974; Whalen et al. 1974; Trump et al. 1976; Ganote et al. 1977; Willerson et al. 1977). After 1 to 2 h of ischaemia, pathological examination reveals cell swelling, dense granules containing calcium within mitochondria and defects in sarcolemmal membrane (Jennings et al. 1964; Korb and Totovic 1969; Kloner et al. 1974; Jennings et al. 1975; Jennings and Ganote 1976). These changes increase upon reperfusion. The prominent feature appears to be a loss of selective membrane permeability which allows the leakage of intracellular enzymes from the cell and the influx of Ca^{++} with extracellular macromolecular components. Such increase in cell membrane permeability to several proteins and macromolecules as a feature of cell damage is well known. Kent (Kent 1967) using a fluorescent antibody technique showed that injured myocardial cells were capable of binding plasma proteins such as albumin, globulins and fibrinogen. With extracellular tracers as lanthanum (Hoffstein et al. 1975), or others (Boutier et al. 1976; Rona et al. 1977), cells with characteristic alterations of severe ischaemic injury failed to exclude the injected substance. However, there has been little information concerning what happens after short-term ischaemia.

In the present study, HRP was used as an extracellular tracer. Perfusion fixation achieved simultaneous fixation of both the membrane and the reaction product (Graham and Karnovsky 1965). After fixation, the tracer could not reach compartments to which it had no access prior to fixation. Controls exclude artefactual absorption of peroxidase of false localization due to diffusion of haemoglobulin in damaged tissue (Karnovsky 1967). Our observations suggest that altered sarcolemmal permeability does not necessarily occur with obvious cellular necrotic changes. After short-term ischaemia, i.e., 15 min in the rat, and 6 min of reperfusion with HRP circulation, the fine strutural diffusion tracer HRP (mol. diam. $\simeq 50$ Å) penetrated the plasma membrane of myocardial cells which have only discrete to moderate structural alterations. Within the section, the cells which failed to exclude the tracer were randomly distributed and the reaction product was evident in some almost normal cells upon ultrastructural examination. However, all cells with more severe structural evidence of ischaemic injury invariably failed to exclude the tracer. After 30 min of ischaemia myocardial cells that showed mitochondrial damage were permeable to HRP. This study of short-term periods of ischaemia, where injury is known to be reversible, indicates that loss of barrier function of sarcolemmal membrane, as revealed by reperfusion, precedes the irreversible cellular degenerative alterations. It is probable that during this early phase of ischaemia, the myocardial cell membrane undergoes changes not detected in the conventional thin sections. Some recent studies using the freeze-fracture technique suggest that rearrangement of intramembranous particles are associated with modifications in the lipid bilayer and with an altered membrane permeability (Ashraf and Halverson 1977).

We have suggested that such changes in the permeability of sarcolemma might be of pathogenic significance in producing post-ischaemic no reflow phen-

omenon (Camilleri et al. 1976). In the capillary bed, alterations in permeability occur at the same time as the blockade of blood flow. It seems likely that myocardial cell oedema and subsarcolemmal bleb formation represent at least one of the mechanisms at work in the capillary collapse and the increase of coronary artery perfusion pressure.

The question whether or not the cell membrane permeability changes may lead to further detrimental events remains unanswered (Leaf 1974; Jennings et al. 1975; Trump et al. 1976; Willerson et al. 1977). It may be that mitochondrial calcium overload is important in the pathogenesis of irreversible myocardial cell injury following coronary occlusion (Kent 1967; Wrogemann and Pena 1976); the mitochondrial deposits of calcium phosphate seem to occur after the cell membrane permeability is altered and increase after reperfusion of ischaemic tissue (Camilleri et al. 1976, Ashraf et al. 1978). Another possibility in that irreversible damage is linked to intra-cellular deposition of a variety of macromolecules which would alter cardiac muscle cell constituents such as contractile proteins and impede cellular reconstitution.

Thus, *membrane permeability* alteration can play a role in acute ischaemic injury by at least *two mechanisms*: blockade of blood flow in the capillary bed, and/or loss of sarcolemmal integrity which could induce degenerative changes of subcellular structures.

At present, our data strongly suggest that jeopardized myocardium could be salvaged by various therapeutic interventions (Maroko et al. 1971) as long as the cell membranes are not irreversibly damaged.

Acknowledgements. We gratefully acknowledge the technical help of Mireille Galtier. Photography: M. Wolfelsperger.

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Accepted May 16, 1980