Diminution of "Reperfusion Injury" in Reperfused Ischemic Myocardium by Phenothiazines. A Quantitative Morphological Study

J. SLEZÁK, N. TRIBULOVÁ, I. GABAUER, A. ZIEGELHÖFFER, V. HOLEC and J. SLEZÁK, Jr.

Institute of Experimental Surgery, Centre of Physiological Sciences, Slovak Academy of Sciences, Dúbravská cesta 9, 842 33 Bratislava, Czechoslovakia

Abstract. The effect of pretreatment by phenothiazines — Chlorpromazine (CPR) /Spofa/ and Trifluoperazine (TFP) /Smith Kline and French/ on reperfusion injury of ischemic myocardium were studied. Reperfusion of ischemic myocardium following an ischemic period exceeding 40 min resulted in morphological, physiological and biochemical changes identical with those induced by enhanced cytosolic Ca^{2+} concentration. Left descending coronary ligation was performed on 70 dogs divided into four group. Group I: permanent occlusion (5 dogs — 60 min, 5 dogs — 120 min, 5 dogs — 180 min); group II: 15 dogs (60 min occlusion + 120 min reperfusion); group III: 20 dogs (60 min occlusion, 15 mg CPR, reperfusion 120 min); group IV: 20 dogs (60 min occlusion, 2 mg TFP + 120 min reperfusion). CPR or TFP were administered 30 min after the ligation. The effect of drugs was quantified on tetrazolium stained gross sections and studied from physiological, biochemical and ultrastructural points of view. Treatment of animals with phenothiazines, known as calmodulin inhibitors, considerably improved the ultrastructure of myocytes in area at risk, and allowed for the recovery of at least 60 per cent of injured myocytes after reflow restoration. Ultrastructural findings tightly correlate with physiological and biochemical results.

Key words: Myocardial infarction — Reperfusion of ischemic myocardium — Phenothiazines — Calmodulin inhibition — Reperfusion injury

Introduction

Myocardial reperfusion after 60 minutes of ischemia, at body temperature, induces biochemical, physiological, and morphological alterations which are

almost identical to changes that occur in the myocardium after short periods of oxygen depletion followed by reoxygenation. This reoxygenation effect ("oxygen paradox") is characterized by disruption of the integrity of the sarcolemma, release and activation of various enzymes, decrease in adenosinetriphosphate (ATP) and creatine phosphate (CP), and increase in the concentration of cytosolic calcium ion (Zimmerman and Hulsmann 1966; Hearse et al. 1973). Cellular damage induced by coronary artery occlusion may be reversible even after relatively long ischemic periods depending on severity of ischemia and on collateral blood flow. Progression of ischemia ultimately results in irreversible necrosis, which occurs first at the inner myocardium with ultimate progression to the periphery. As the necrosis progresses, areas of myocardium which are potentially salvageable become also injured.

Of the many hypotheses about the mechanism of cellular damage during ischemia and reperfusion, those most plausible are based on the concept of degradative changes in membrane phospholipids. Membrane phospholipids are necessary for cellular integrity (Davson and Danielli 1952) and participate in the formation of organelles and other components; they are also a part of membrane bound enzymes (Fleischer et al. 1962; Kavanau 1963).

Structural changes in phospholipids are immediately reflected in abnormal cell metabolism. There are thought to be four possible mechanisms responsible for abnormal degradation of membrane phospholipids, thereby leading to membrane damage during ischemia.

The first hypothesis suggests that metabolic products that are normally found in cells and have detergent effects, may excessively accumulate in ischemia (Idell-Wenger et al. 1978; Shug et al. 1978; Whitmer et al. 1978; Adams et al. 1980).

A second theory suggests activation of endogenous membrane-bound and calcium- and calmodulin-dependent phospholipases by increased cytosolic calcium ion accumulation during postischemic reperfusion (Weglicki et al. 1971; Van der Bosch 1974).

A third concept, related to the previous two, suggests that there is release and activation of lysosomal enzymes either from myocytes or, subsequently, from leukocytes which are chemotactically attracted to the ischemic region. These enzymes then cause degradation of membrane phospholipids (Topping and Travis 1974; Wildenthal 1975; Hoffstein et al. 1976; Decker and Wildenthal 1978; Adams et al. 1980; Grinde 1982).

Finally, it has been proposed that peroxides and hydroxyl radicals are formed after ischemia, and these cause irreversible damage (Hansson et al. 1983; Hertz and Cloarec 1984).

Disruption of lysosomal membrane has been demonstrated 30 minutes after onset of ischemia (Decker et al. 1977; Welman and Peters 1977a; 1977b). In

addition, activation of lysosomal enzymes was described after 60 minutes of ischemia (McCallister et al. 1978). Allan and Welman (1980) found that proteases released by cardiac myocytes are also able to degrade mitochondrial proteins. Almost all these calcium dependent proceses are also calmodulin dependent.

Prompted by the above hypotheses, we attempted to inhibit the activation effect of high cytosolic Ca^{2+} concentration on various endogenous calmodulin dependent degrading enzymes (Busch et al. 1972; Toyo-oka et al. 1977; Beckman et al. 1982; Slezák et al. 1983b; Slezák et al. 1984a; 1984b) by directly inhibiting calmodulin, and thereby protecting the ischemic heart muscle from irreversible damage during reperfusion.

We have previously shown that experimental doses of Chlorpromazine (CPR) or Trifluoperazine (TFP) as calmodulin inhibitors and membrane stabilizers do not cause any cellular injury in normal nonischemic myocardium (Slezák et al. 1982).

In the present work we employed quantitative histochemical, ultrastructural and biochemical methods to evaluate the protective effects of phenothiazines (CPR and TFP) on ischemic reperfused myocardium.

Materials and Methods

Quantitative morphological studies: A total of 70 mongreal dogs of both sexes, average weight 20 kg, were used; they were given 2 mg/kg b.w. of heparin intravenously. Anesthesia was induced with 30 mg/kg b.w. penthotal and oxygenation was maintained by endotracheal intubation and controlled mechanical respiration.

The chest was opened in the 4th intercostal space between the left 4th and 5th ribs. Physiologic monitors were applied, as described below. The left anterior descending coronary artery was ligated between the first and second diagonal branches. Four groups of experiments were performed.

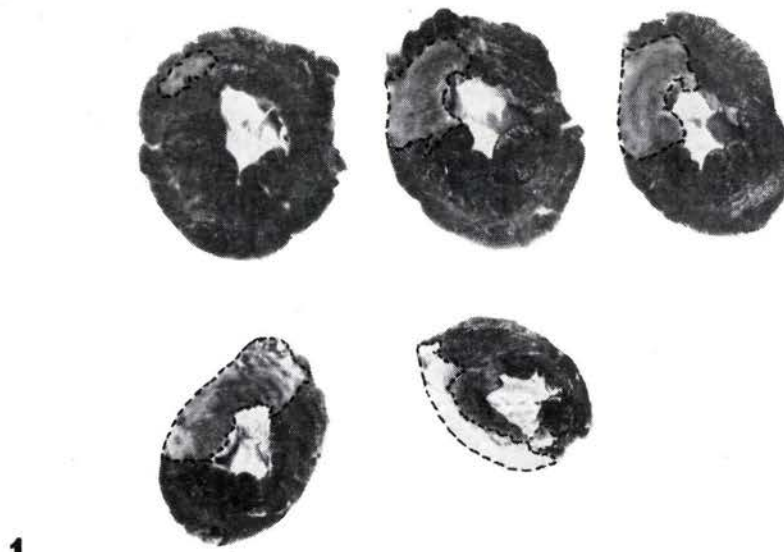
Group I: permanent occlusion (60 minutes of permanent occlusion, 5 dogs; 120 minutes — 5 dogs; 180 minutes — 5 dogs)

Group II: 60 min of permanent occlusion followed by 120 min of reperfusion (15 dogs)

Group III – IV: 60 min occlusion (20 + 20 dogs) followed by 120 min of reperfusion with following treatment:

CPR (15 mg/kg b.w.) /group III/ or TFP (2 mg/kg b.w.) /group IV/ were administered intravenously 30 minutes after coronary artery occlusion. Thirty minutes later, reperfusion was started and mantained for 120 minutes.

Following the reperfusion period, the heart was removed and immediately examined. Transversal 1 cm sections were cut from the left ventricle. Samples for histochemistry, electron microscopy and biochemistry were taken from a) subepicardial (reperfused) areas, b) the border area between infarcted and reperfused zone, and c) the center of the infarction zone. Thick sections were incubated in 0.005 % NBT (Nitroblue tetrazolium) (Figs. 1 and 2) to vizualize ischemic areas, and were then examined and recorded utilizing a transparent sheet for direct measuring. The relative volumes of ischemic and non ischemic myocardial tissue were calculated using the standard planimetric techni-



1

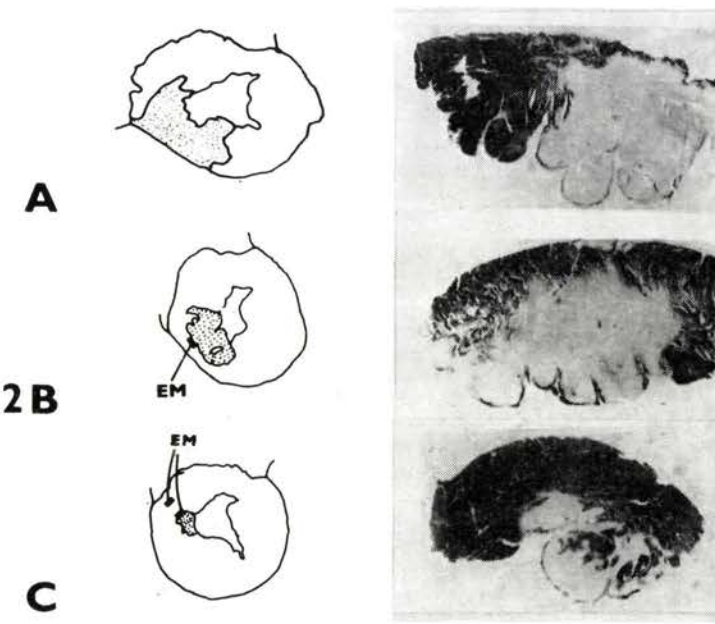


Fig. 1. Transverse thick sections of left ventricle incubated in NBT solution to visualize infarcted area (pale).

Fig. 2. Sections across the left heart ventricle recorded on transparent sheets. *Left*: Dotted areas show infarcted myocardium. *Right*: Low-magnification microphotographs of cryostate sections of the same heart incubated for α-glucan phosphorylase to visualize ischemic areas. *A*: transmural

que by one of the authors; the specimens were encoded to prevent the examiner from identifying the type of the experiment.

For electron microscopy 1 mm^3 pieces of tissue were immediately immersed in 3 per cent glutaraldehyde in 0.1 mmol . l^{-1} cacodylate buffer and fixed for 3 hours. After postfixation in 1 per cent OsO_4 the tissue was embedded in Epon 812. Thick sections (1 μm) and light microscopy examination were used to select areas of infarcted, reperfused and normal myocardium. Thin sections were double stained with heavy metals and studied in a Tesla 500 transmission electron microscope.

Histochemical studies were performed on samples which included both infarcted and normal nonischemic myocardium. The samples were quickly frozen in isopentane cooled to −70 °C. Seven μm thick cryostat sections were incubated in a medium to demonstrate tetrazolium reductases (Fig. 1) and α-glucan phosphorylase (Fig. 2, right).

Physiologic studies were performed on all dogs used for morphologic studies. Parameters monitored every 30 minutes included aortic blood pressure, aortic blood flow (Nycotrone electro magnetic head) and systolic and end-diastolic left ventricular pressure. Left vertricular dp/dt max and V_{max} were calculated from the left ventricular pressure. Also calculated were cardiac index, systolic volume index, systemic resistance index and left ventricular minute work index.

Myocardial blood flow and its distribution were measured by the Sapirstein method using radioactive rubidium captation in additional 15 experiments performed under exactly the same conditions (as groups I—III). At the end of the experiments, 1.85 MBq ^{86}RbCl was injected through a jugular vein catheter. Sixty seconds later the heart was removed and divided into ischemic, nonischemic, and marginal zones as described above. Each of these were further divided into subendocardial and subepicardial layers. Activities of samples were measured on an automatic gamma counter. Results were expressed as percentage of total activity per gram of tissue. By multiplying these values with cardiac output, regional myocardial blood flow (in ml . min . g^{-1} tissue) was calculated (Table 1, Fig. 3). The endo/epi ratio was calculated from the determined values of endocardial and epicardial blood flow.

Biochemical studies: 35 dogs were studied under exactly the same experimental conditions as those used for morphological studies. Samples from nonischemic parts, periphery and center of the ischemic areas as well as from reperfused area, were taken from beating hearts using the freeze clamp technique. Metabolites including all adenine nucleotides, lactate and pyruvate were determined in tissue extracts enzymatically on the principle of Warburg's optical test as described elsewhere (Šiška et al. 1974).

infarction after 3 hours of permanent ligation. *B*: after one hour ligation and two hours of reperfusion. Note relatively thick subepicardial layer salvaged by reperfusion. *C*: 1 hour ligation of the coronary artery followed by 2 hours of reperfusion of the heart pretreated with CPR (15 mg per kg b.w. administred 30 min after ligation). Note the large area of salvaged tissue in *C* compared with *B* and *A*. EM — areas where tissue samples for electron microscopy and biochemistry were taken from.

Table 1. Regional myocardial blood flow ($ml \cdot min^{-1} \cdot g^{-1}$)

		Occlusion 3 h	Occlusion 1 h + Reperfusion 2 h	Occlusion 1 h + CPR + Reperfusion 2 h
Ischemic zone	epi	0.109 ± 0.007	0.400 ± 0.008^a	0.606 ± 0.018^b
	endo	0.103 ± 0.007	0.346 ± 0.004^a	0.548 ± 0.019^b
	endo/epi	0.94 ± 0.03	0.87 ± 0.02^a	0.91 ± 0.05^b
Border zone	epi	0.200 ± 0.010	0.538 ± 0.011^a	0.669 ± 0.017^b
	endo	0.192 ± 0.010	0.462 ± 0.005^a	0.595 ± 0.017
	endo/epi	0.96 ± 0.03	0.86 ± 0.04^a	0.89 ± 0.05^b
Nonischemic zone	epi	0.522 ± 0.018	0.714 ± 0.011^a	0.742 ± 0.012
	endo	0.539 ± 0.018	0.761 ± 0.009^a	0.776 ± 0.018
	endo/epi	1.03 ± 0.04	1.07 ± 0.06	1.05 ± 0.06

Mean ± Sem
a $p < 0.05$ between group I and II
b $p < 0.05$ between group II and III

epi = subepicardial layer
endo = subendocardial layer

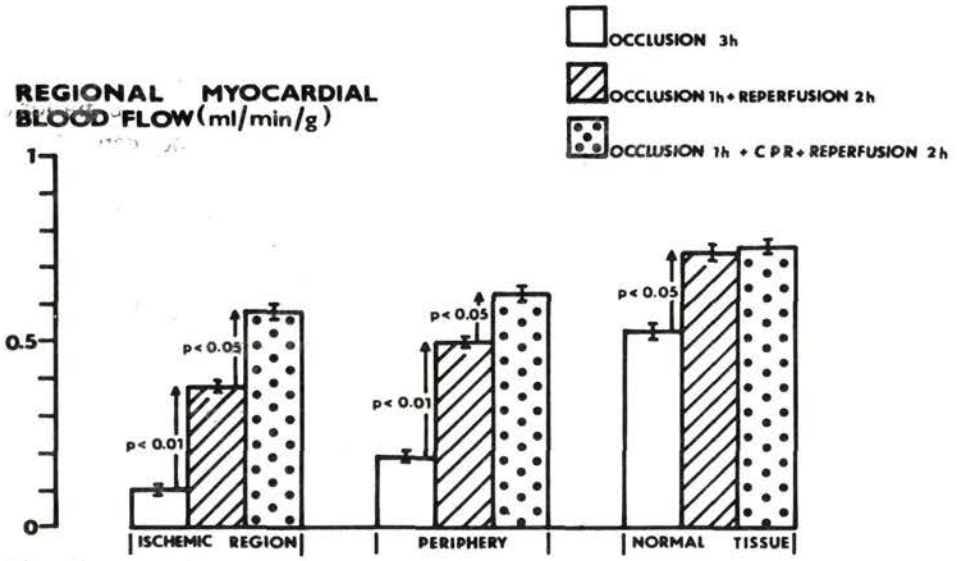

REGIONAL MYOCARDIAL BLOOD FLOW (ml/min/g)
OCCLUSION 3h
OCCLUSION 1h + REPERFUSION 2h
OCCLUSION 1h + CPR + REPERFUSION 2h
1
0.5
0
p< 0.01
p< 0.05
p< 0.01
p< 0.05
p< 0.05
ISCHEMIC REGION
PERIPHERY
NORMAL TISSUE

Fig. 3

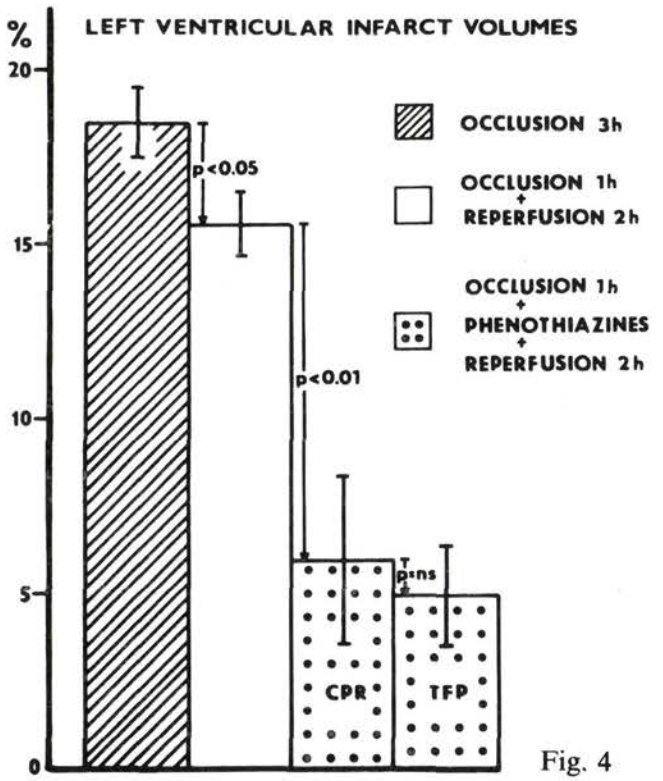

%
LEFT VENTRICULAR INFARCT VOLUMES
20
15
10
5
0
OCCLUSION 3h
OCCLUSION 1h + REPERFUSION 2h
OCCLUSION 1h + PHENOTHIAZINES + REPERFUSION 2h
p<0.05
p<0.01
p=ns
CPR
TFP

Fig. 4

Results

Quantitative morphological study: Reaction with NBT clearly visualized and differentiated between ischemic and nonischemic tissue (Fig. 1). Permanent occlusion (group I) resulted in infarction of an average of 18.5 per cent of the left ventricle (Figs. 2*A* and 4). After reperfusion (group II), the infarcted area was 15.6 per cent, with most of the subepicardial layer being spared (area of the largest collateral flow) (Fig. 2*B*). In the central and subendocardial areas, reperfusion caused accentuation of ischemic changes and enhanced destruction of myocytes (Fig. 5). Pretreatment with phenothiazines (groups III and IV) reduced the area of infarction to 5—6 per cent of the left ventricular mass (Figs. 2*C* and 4).

Electron microscopy: Myocardial cells from hearts with permanent occlusion, contained mitochondria with numerous amorphous densities in the border zone and in reperfused subepicardial layer (groups I and II) (Figs. 6 and 7), 120—300 nm in size. Ischemic alterations, including descreased density of the matrix, membrane abnormities, and interfibrillar edema, were easily recognized (Figs. 7 and 8). Affected myocytes were contracted and showed dissolution of intercalated discs (Fig. 8).

Reperfused myocardium of phenothiazine treated animals (groups III and IV) showed changes of various degrees such as inter- and intracellular edema, spotty vacuolation of mitochondria, dense and contracted mitochondria (Fig. 9*A*), diminished amount of glycogen, and increassed number of myelin-like bodies (Fig. 9*B*).

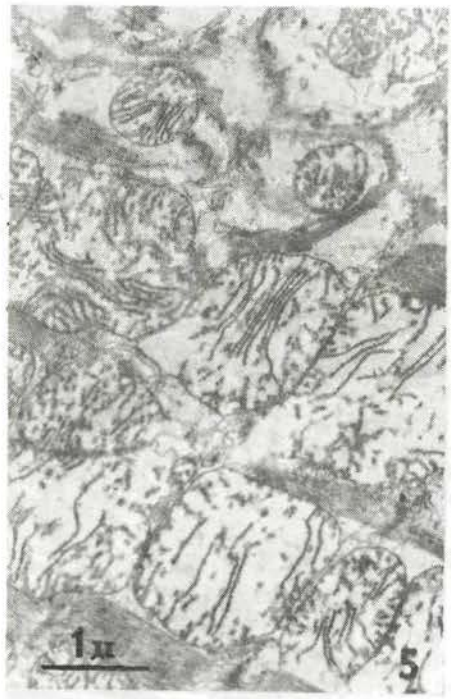
A prominent feature was fusions of as many as 5 mitochondria, creating giant mitochondria in which the margins of the original mitochondria could be still recognized (Fig. 10*A* and *B*). No nuclear changes could be detected (Fig. 9*A*).

Fig. 5. Reperfusion resulted in enhanced destruction of myocytes in severely ischemic subendocardial layer. Mitochondria are edematous with ruptures and/or fragmentations of outer and inner membranes. Electron micrograph — magnification — the bars represent 1 μm.

Fig. 6. The reperfused subepicardial layer contained mitochondria with numeros amorphous densities, and a decreased density of the matrix. Sarcomeres are dilated with degradation changes in I bands.

Fig. 7. Border zone of a permanently occluded coronary artery with spontaneous reperfusion after 2 hours of reperfusion. Ultrastructural changes are identical with changes shown in Fig. 6.

Fig. 8. Reperfused border zone with contracted sarcomeres and mitochondrial changes, amorphous densities present. Dehiscence and dissolution of the intercalated disc is a frequent prominent feature in reperfused hearts.


1μ
5


6


7


8

Histochemistry: Cryostat sections incubated in a medium for α-glucan phosphorylase (Fig. 2, right) showed reactions identical to those of the macrosections incubated in media containing NBT.

Myocardium that stained with NBT also stained positively after incubation for α-glucan phosphorylase. The reaction with NBT clearly differentiated between ischemic and nonischemic tissues (Fig. 1) in the time span used in our experiments.

Physiologic studies: Myocardial contractility and cardiac performance were negatively affected by reperfusion (Table 2, 3, Fig. 11). There were significant decreases of mean aortic pressure, dp/dt max, V_{max} cardiac index, systemic volume index and left ventricular minute work index, and significant increase in left ventricular end-diastolic pressure. Arrhythmias were documented in 95 per cent of the postperfusion animals. In the phenothiazine pretreatment groups there was a reduction of left ventricular end-diastolic pressure, mean aortic pressure and systemic resistance index, and concomittant increases in dp/dt max, V_{max} and cardiac index. Arrhythmias were observed in only 16 per cent of animals. There were no significant differences between the action of CPR and TFP, although the TFP-treated animals showed a slower onset of the effect, the vasodilatation effect was weaker, and cardiac function values were generally reduced (Tables 2 and 3, Figs. 11 and 12) as compared to the CPR group.

After 3 hours of occlusion the regional myocardial blood flow (Table 1, Fig. 3) was significantly reduced: by 83 per cent in the ischemic areas, by 67 per cent in the border zone, and by 17 per cent in nonischemic myocardium. In the group undergoing 60 minutes occlusion, followed by 2 hours reperfusion, the regional myocardial blood flow increased dramatically; by 251 per cent in ischemic region, by 155 per cent in border zone, and by 39 per cent in normal nonischemic region. The increase was most marked in the subepicardial layer as documented by a decreased endo/epi ratio in ischemic and border zone (Table 1).

After CPR pretreatment, the regional myocardial blood flow increased by 55 per cent in the ischemic zone and by 26 per cent in the border zone as compared to non CPR reperfusion myocardium. Blood flow redistribution in the subendocardial layer was affected in a similar manner. There was an only 5 per cent

Fig. 9. *A,B*: Reperfused CPR treated myocardium. The ischemic subepicardial layer showed a relatively well preserved ultrastructure after two hours of reperfusion. The main feature are dark mitochondria of various size and decreased amounts of glycogen granules.

Fig. 10. *A,B*: Electronograms from the same experiment as showed on Figs. 7. and 8. Another prominent feature of reperfused reversibly injured tissue are fusions of mitochondria with still recongnizable original margins. Slight interfibrilar oedema is also present. *B*: Mitochondria show slight changes in the arrangement of cristae and spotty vacuolation.

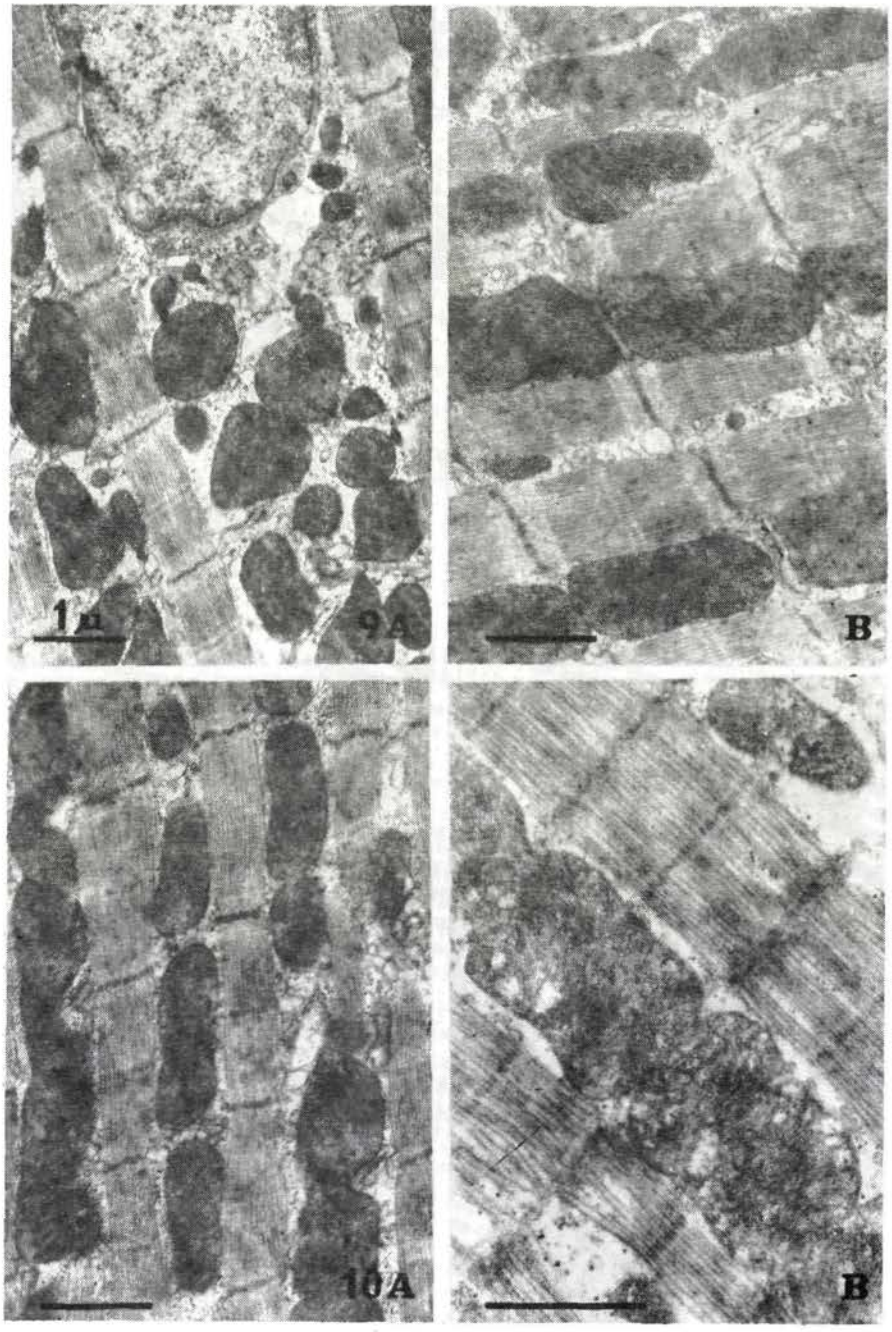

1μ
9A
B
10A
B

Table 2. Myocardial contractility

Group $N = 20$		dp/dt max, (mmHg/s)	V_{max} (m/s)	LVEDP (mmHg)	MAP (mmHg)
Occlusion + Reperfusion	0′	1822.5 ± 119.5	0.94 ± 0.03	7.0 ± 0.4	148.3 ± 6.5
	60′	1530.0 ± 108.4[a]	0.80 ± 0.03[a]	13.1 ± 0.7[a]	128.3 ± 4.8
	180′	1507.5 ± 73.3[a]	0.74 ± 0.03[a]	13.8 ± 0.7[a]	120.0 ± 2.6[a]
Occlusion + Reperfusion + + CPR	0′	1927.1 ± 100.0	0.98 ± 0.02	7.4 ± 0.5	150.0 ± 4.2
	60′	1620.0 ± 61.0[a]	0.80 ± 0.03[a]	14.9 ± 0.7[a]	102.4 ± 3.2[a]
	180′	1807.3 ± 102.0[b]	ýř0.93 ± 0.02[b]	8.7 ± 0.5[b]	86.3 ± 5.3[ab]
Occlusion + + Reperfusion + TFP	0′	1884.0 ± 49.0	1.07 ± 0.04	7.1 ± 0.1	146.0 ± 3.1
	60′	1536.0 ± 22.4[a]	0.83 ± 0.01[a]	13.7 ± 0.2[a]	116.0 ± 3.7[a]
	180′	1684 ± 45.8[c]	0.95 ± 0.04[c]	8.7 ± 0.1[c]	92.0 ± 3.3[ac]

Mean ± Sem

a $p < 0.05$ against the preocclusive values
b $p < 0.05$ between group I and II
c $p < 0.05$ between group I and III
d $p < 0.05$ between group II and III

LVEDP = left ventricular enddiastolic pressure
MAP = mean arterial pressure

Table 3. Cardiac performance

Group $N = 20$		CI (ml/min/kg)	SVI (ml/syst/kg)	SRI (dyn/s/cm^2/kg)	LVMWI (kgm/min/kg)
Occlusion + Reperfusion	0′	71.1 ± 3.9	0.39 ± 0.02	169.5 ± 7.3	143.3 ± 7.1
	60′	59.5 ± 3.0[a]	0.31 ± 0.01[a]	173.0 ± 10.1	103.0 ± 7.1[a]
	180′	55.9 ± 3.2[a]	0.25 ± 0.01[a]	167.9 ± 5.9	93.2 ± 6.4[ab]
Occlusion +	0′	75.2 ± 4.4	0.43 ± 0.02	162.2 ± 8.7	146.7 ± 6.4
+ Reperfusion + CPR	60′	61.6 ± 4.7[a]	0.31 ± 0.01[a]	118.4 ± 5.8[a]	75.5 ± 5.5[a]
	180′	69.9 ± 5.0[b]	0.35 ± 0.02[b]	100.5 ± 6.4[b]	88.9 ± 5.0[a]
Occlusion +	0′	74.7 ± 1.4	0.43 ± 0.01	149.0 ± 3.4	141.4 ± 4.4
+ Reperfusion + TFP	60′	60.6 ± 1.2[a]	0.33 ± 0.01[a]	135.5 ± 5.7	84.3 ± 3.2[a]
	180′	66.9 ± 0.7[c]	0.36 ± 0.01[c]	99.9 ± 4.4[ac]	75.7 ± 2.8[ac]

Mean ± Sem

a $p < 0.05$ against the precclusive values
b $p < 0.05$ between group I and II
c $p \pm 0.05$ between group I and III
d $p \pm 0.05$ between group II and III

CI = cardiac index
SVI = systolic volume index
SRI = systemic resistance index
LVMWI = left ventricular minute work index

increase in the nonischemic tissue, without any significant changes in redistribution (Fig. 3, Table 1).

Biochemical studies: Acute coronary occlusion (60 min) induced a considerable decrease in adenine nucleotides and increase in lactate and partially also in

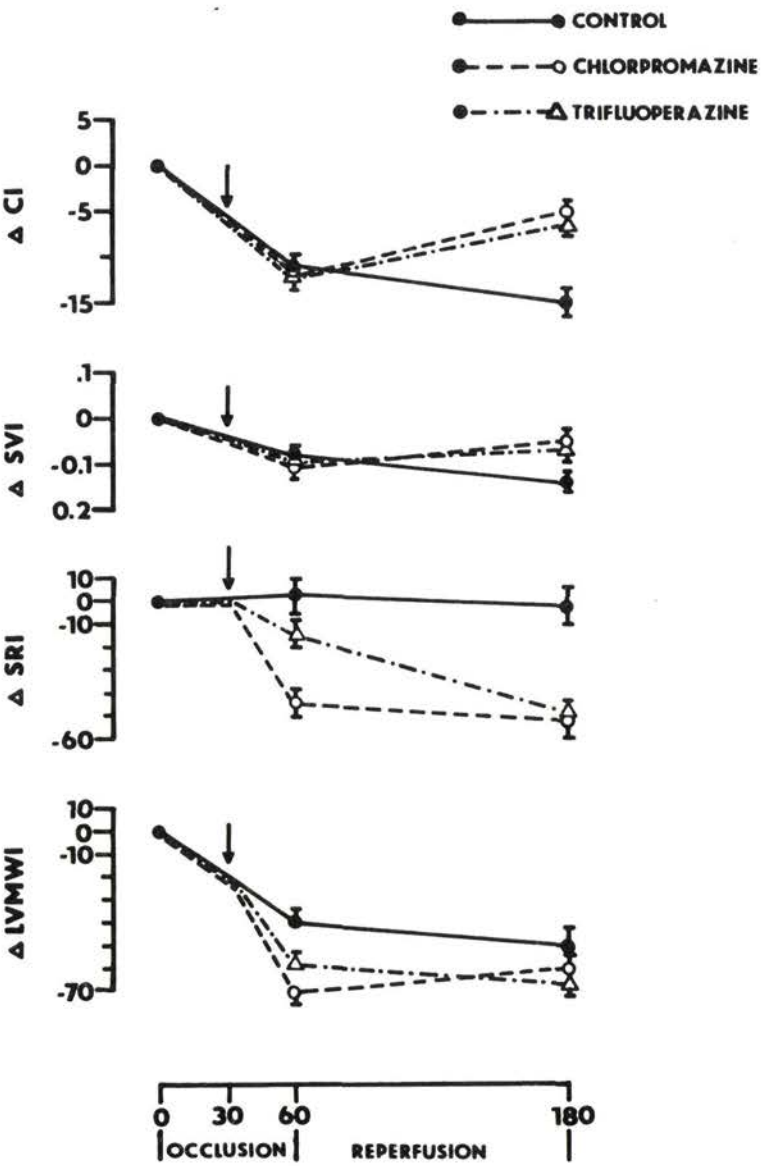


Fig. 11. Hemodynamic parameters characterizing contractility of the myocardium.

pyruvate in both the ischemic and nonischemic areas of the left ventricle (Fig. 7) (groups II and III). After reperfusion these changes were even more dramatic (Table 4) (group IV). In contrast, a slight but in significant improvement in adenine nucleotide contents and especially in the pyruvate lavel was observed in

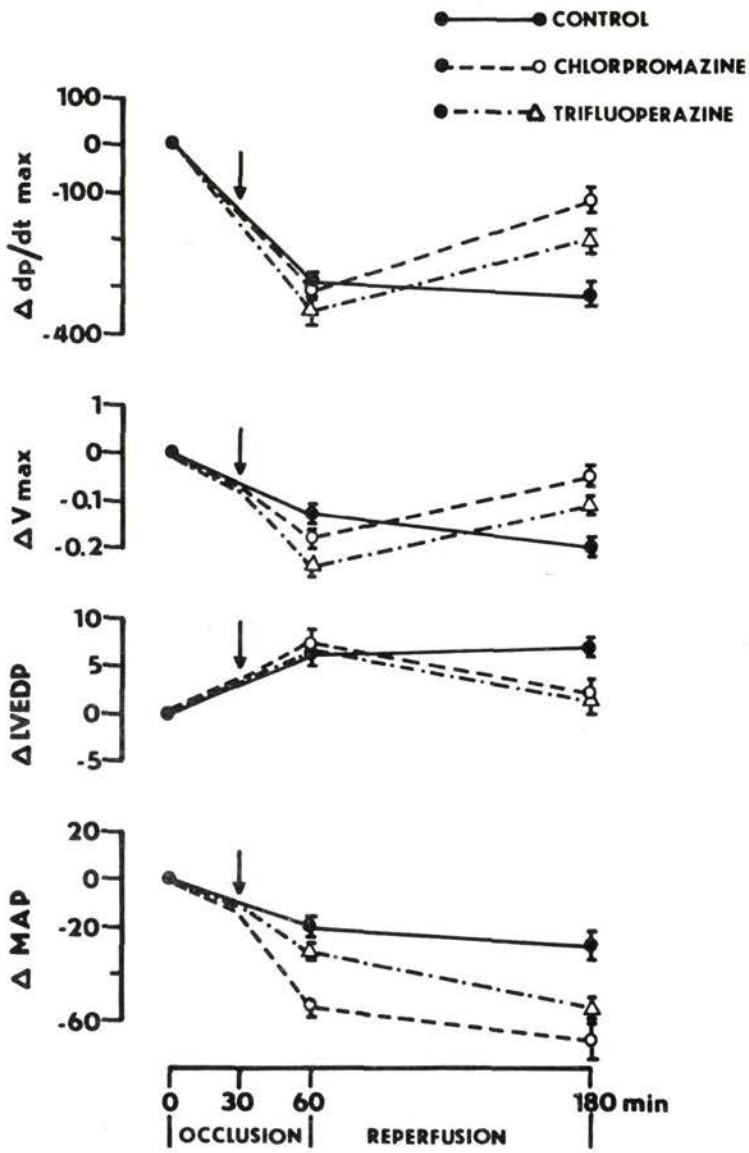


Fig. 12. Hemodynamic parameters characterizing the heart performance.

Table 4.

Group	No	ATP	ADP	AMP	ADN	LAC	PYR
		μmol . g^{-1} wt . w.					
Control hearts in situ $N = 20$	I	5.53 ± 0.57^{d}	1.24 ± 0.15^{d}	0.31 ± 0.08^{c}	7.16 ± 0.36^{d}	1.72 ± 0.27^{d}	0.07 ± 0.02^{d}
Occlusion, nonischem. L. V. $N = 10$	II	3.45 ± 0.29^{a}	0.55 ± 0.27^{b}	0.66 ± 0.10^{ad}	4.66 ± 0.25^{b}	3.78 ± 0.16^{b}	0.07 ± 0.03^{d}
Occlusion, ischem. L. V. $N = 10$	III	3.00 ± 0.67^{a}	0.58 ± 0.24^{b}	0.60 ± 0.22^{d}	4.18 ± 0.27^{b}	20.20 ± 2.83^{bd}	0.24 ± 0.07^{b}
Occlusion, reperf. ischem. L. V. $N = 5$	IV	2.80 ± 0.61^{b}	0.76 ± 0.10^{b}	0.12 ± 0.04^{b}	3.66 ± 0.73^{b}	4.31 ± 1.66^{a}	0.22 ± 0.05^{b}
Occlusion trifluoperazine, reperf. ischem. L. V. $N = 5$	V	4.70 ± 0.18^{d}	0.82 ± 0.05^{b}	0.17 ± 0.02^{a}	5.69 ± 0.14^{bd}	3.10 ± 0.35^{a}	0.13 ± 0.01^{bc}
Occlusion, trifluoperazine, reperf. ischem. L. V. $N = 5$	VI	1.78 ± 0.22^{bc}	0.48 ± 0.04^{bc}	0.12 ± 0.03^{b}	2.37 ± 0.39^{bd}	2.85 ± 0.39^{a}	0.19 ± 0.02^{b}
Occlusion, chlorpromazine, reperf. ischem. L. V. $N = 6$	VII	3.68 ± 0.42^{a}	1.02 ± 0.08^{c}	0.35 ± 0.14^{c}	4.97 ± 0.57^{b}	4.01 ± 0.95^{a}	0.13 ± 0.03^{b}

Results are means $\pm$ S. E. M. ADN — the total pool of adenine nucleotides
$a < 0.05$ and $b < 0.01$ agaist the control hearts
$c < 0.05$ against group IV
$d < 0.05$ against group IV
Tissue contents of adenine nucleotides, lactate and pyruvate in non-ischemic and ischemic areas of the left ventricle after acute coronary occlusion with and without chlorpromazine or trifluoperazine treatment and subsequent reperfusion.

ischemic tissue of the CPR-treatment and reperfused group (Table 4, VII). TFP was shown to have clearly benefical effects, in the nonischemic tissue only (Table 4, V and VI).

Discussion

We could show that a single phenothiazine dose given 30 min after occlusion of the left descending coronary artery, significantly protects the myocardium from the effect of ischemia, particularly the bordering region, separating the center of the ischemic focus from normal myocardium. There was no significant effect on the irreversibly damaged central area of ischemic tissue (the least perfused tissue during occlusion).

Ischemia caused by coronary artery occlusion induces profound changes in myocytes. The quality and quantity of these changes depend on the duration of ischemia and on the degree of collateral flow.

Prolonged ischemia induces anaerobiosis with subsequent deterioration of the energy metabolism. This is characterized by decrease of ATP, ADP and AMP levels i.e., a depletion of the entire adenine nucleotide pool with a concomittant increase in lactate and pyruvate levels in the myocardial tissue (Ziegelhöffer et al. 1976). These alterations occur not only in the ischemic regions, but also involve the well perfused but work-overloaded adjacent areas of the ventricle (Fedelešová et al. 1978; Slezák et al. 1983a). In confirmation of previous experiments (Ziegelhöffer et al. 1979), reperfusion made the coronary occlusion-induced metabolic disorders even more pronounced. This detrimental effect could be partially abolished by the administration of CPR or TFP. CPR had the greatest effect in the ischemic areas whereas the TFP response was best seen in nonischemic areas of the infarcted left ventricle.

During the first 20 min of normothermic ischemia the changes are reversible (Herdson et al. 1965; Slezák 1969; Shen and Jennings 1972a; 1972b; Jennings and Ganote 1974). If the blood flow is restored after this period, aerobic metabolism is also restored. However, if ischemia is prolonged, there are more irreversible changes which are apparently dependent on the residual blood flow in the ischemic area. Reperfusion of ischemically damaged cells results in accelerated and irreversible changes at the ultrastructural level, and in cell death. After 20 minutes of total ischemia the number of dead cells is minimal (Jennings et al. 1978). After 30 minutes fewer than 30 per cent are dead; however after 45 minutes about 75 per cent, and after 60 minutes 100 per cent of cells are irreversibly damaged. These differences reflect the adequacy of collaterals, and in the model of occlusion in vivo, the residual blood flow. In dogs the number of collaterals is very variable (Slezák, unpublished data).

Intracellular calcium is enormously increased in tissue ischemic for 40 minutes and then subjected to 20 minutes of reperfusion (Jennings et al. 1964; Shen and Jennings 1972a; 1972b; Whalen et al. 1974). Calcium is thought to cause contraction bands (Herdson et al. 1965; Kloner et al. 1974a; 1974b; Slezák and Tribulová 1975a; 1975b), and crystalline and granular deposits are characteristically seen in mitochondria of ischemically and irreversibly damaged tissue after reperfusion (Jennings and Hawkins 1980).

Changes typical of long term (150 minutes) ischemia (Kloner et al. 1974b) are seen when shorter period of total ischemia (with irreversible changes) are followed by reperfusion.

During ischemia high energy phosphate stores (HEP) get exhausted (Jennings and Hawkins 1980), cytosolic Ca^{2+} concentration increases, and myocyte and sarcolemmal calcium-dependent enzymes including proteases and phospholipases are activated. These calcium dependent enzymes are also mostly calmodulin dependent, and degrade all myocyte components including membrane phospholipids. The membranes are then unable to maintain a high concentration gradient of Ca^{2+} between the extracellular and intracellular space. After reperfusion, the amount of intracellular Ca^{2+} increases to concentrations (Shen and Jennings 1972a; 1972b) that activate various degradation enzymes (also destroying the membranes). The damaged plasmalemma allows calcium to enter the myocyte thereby maintaining a vicious circle causing the death of the cell (Hearse 1977).

In previous experiments, the extent of damage to selective permeability of the sarcolemma was confirmed by treating the tissue with colloidal lanthanum (Slezák et al. 1982). In the ischemia-reperfusion experiments the sarcolemma was ruptured, the myocytes destroyed, and intracellular granules of La which entered the cells were found (Slezák et al. 1982).

Schanne et al. (1979) showed that disruption of the sarcolemma itself, without high Ca^{2+} concentration, will not lead to the death of the cell.

Chlorpromazine can affect membranes in several ways; it can inhibit membrane permeability for Ca^{2+} (Seeman 1972). We have already mentioned that ischemia results in degradation of membrane phospholipids (Chien et al. 1980) prior to loss of myocyte viability (Schanne et al. 1980). CPR affects phospholipid metabolism and prevents degradation of membrane phospholipids (Chien et al. 1979) allowing the membranes to maintain their function as a selective barrier to Ca^{2+}. However, phenothiazines also have calmodulin inhibitory effect, and limit Ca^{2+} binding to calmodulin. Ca^{2+} is then unable to activate many calmodulin dependent processes; we believe that this effect is most important in preventing reperfusion injury.

Different other mechanisms can also play important roles. Increased degradation of phospholipids in ischemic cells contributes to the accumulation of

metabolic intermediates, such as ester of acyl CoA, acylcarnitin etc. (Idell-Wenger et al. 1978; Shug et al. 1978; Whitmer et al. 1978). These have a membrane detergent effect. The activation of lysosomal enzymes may also play a role in damaging membranes (Wildenthal 1975; Hoffstein et al. 1976; Decker et al. 1977) during ischemia and reoxygenation (Ingwal et al. 1975). However, some studies have documented that release of lysosomal enzymes does not contribute to the phospholipids degradation (Kane et al. 1980), and that increased cytosolic Ca^{2+} concentration itself does not influence the degradation of lipids by activating membrane phospholipases either (Van der Bosch 1974; Farber et al. 1981).

So far, CPR has been used in experiments in very high doses to protect membranes from ischemic injury. In clinical practice, it has been used in therapeutic doses in premedication of patients, and it has been thought to have a protective effect on liver cells (Chien et al. 1977).

In our previous experiments we tried to inhibit proteases by aprotinine. A marked protective effect was observed only when aprotinine was used in combination with phenothiazines, or when it had been given alone after prolonged periods of ischemia (Slezák et al. 1982), when it could inhibit proteases released from immigrated leukocytes. High doses of chlorpromazine can also have negative effects on cell metabolism and contribute to ultrastructural alterations. Rats given 50 mg/kg b.w. of chlorpromazine for several days showed distinct ultrastructural changes in skeletal muscle and associated altered physiological functions (Saito et al. 1982). However, a single dose of 30 mg/kg b.w. did not cause any significant changes in cardiac myocyte ultrastructure (Slezák et al. 1982).

In the present experiments, we tried relatively low doses of CPR (15 mg/kg b.w.) and TFP, and administered them after coronary artery occlusion. We chose the interval of 30 min because of its being within the range of reversibility of myocardial ischemic changes, and because of its possible relevance to the clinical setting. The beneficial effect of CPR, and TFP when given 30 min after coronary artery occlusion, was seen both in terms of improved physiological functions and in that of a reduction of the zone of infarction. Phenothiazines may also help to reduce the likelihood of shock. The potential clinical application to human coronary heart dissease are obvious and, on the basis of our results, clinical trials may be justifiable.

References

Adams R. J., Cohen D. W., Gupte S., Johnson J. D., Wallick E. T., Wang T., Schwartz A. (1980): In vitro effects of palmitylcarnitine on cardiac plasma membrane Na-K-ATPase and sarcoplasmic reticulum Ca^{2+}-ATPase and Ca^{2+} transport. J. Biol. Chem. **255,** 4519—4527

Allan R. M., Welman E. (1980): Proteolysis of isolated mitochondria by myocardial lysosomal enzymes. Biochem. J. **190,** 139—144

Beckman J. K., Owens K., Knauer T. E., Weglicki W. E. (1982): Hydrolysis of sarcolemma by lysosomal lipases and inhibition by chlorpromazine. Amer. J. Physiol. (Heart Circ. Physiol. 11) **242,** H652—H656

Busch W. A., Stromer M. M., Goll D. E., Suzuki A. (1972): Ca^{2+} — specific removal of Z-lines from rabbit skeletal muscle. J. Cell. Biol. **52,** 367—381

Chien K. R., Abrams J., Pfau R. G., Farber J. L. (1977): Prevention by chlorpromazine of ischemic liver cell death. Amer. J. Pathol. **88,** 539—556

Chien K. R., Abrams J., Klein M. S., Seroni A., Martin J. T., Farber J. L. (1978): Accelerated phospholipid degradation and associated membrane dysfunction in irreversible, ischemic liver cell injury. J. Biol. Chem. **253,** 4809—4817

Chien K. R., Pfau R. D., Farber J. L. (1979): Ischemic myocardial cell injury: Prevention by chlorpromazine of an accelerated phospholipid degradation and associated membrane dysfunction. Amer. J. Pathol. **97,** 505—529

Davson H., Danielli J. P. (1952): The Permeability of Natural Membranes, Cambridge University Press, Cambridge

Decker R. S., Wildenthal K. (1978): Sequentional lysosomal alterations during cardiac ischemia. II. Ultrastructural and cytochemical changes. Lab. Invest. **38,** 662—673

Decker R. S., Poole A. R., Griffin E. E., Dingle J. T., Wildenthal K. (1977): Altered distribution of lysosomal cathepsin D in ischemic myocardium. J. Clin. Invest. **59,** 911—921

Farber J. L., Chien K. R., Mittnacht S., Jr. (1981): The pathogenesis of irreversible cell injury in ischemia. Amer. J. Pathol. **102,** 2, 271—281

Fedelešová M., Ziegelhöffer A., Styk J., Slezák J. (1978): Effect of myocardial ischemia on metabolic adaptability in the dog heart. I. In: Artifical Circulation (Ed. J. Slezák) pp. 189—200, VEDA, Bratislava

Fleischer S., Brierley G., Klouwen H., Slatterback D. E. (1962): Studies of the electron transfer system. The role of phospholipids in electron transfer. J. Biol. Chem. **237,** 3264—3272

Grinde B. (1982): Selective inhibition of lysosomal protein degradation by the thiol proteinase inhibitors E-64, Ep-459 and Ep-475 in isolated rat. Biochim. Biophys. Acta **701,** 328—333

Hansson R., Hansson O., Lundstam S., Petersson S., Schierstén T., Weldenström J. (1983): Effects of free radical scavenergs on renal circulation after ischemia in the rabbit. Clin. Sci. **65,** 605—610

Hearse D. J., Humphrey S. H., Chain E. B. (1973): Abrupt reoxygenation of the anoxic potassium-arrested perfused rat heart: A study of myocardial enzyme release. J. Mol. Cell. Cardiol. **5,** 395—407

Hearse D. J. (1977): Reperfusion of the ischemic myocardium. J. Mol. Cell. Cardiol. **9,** 605—616

Herdson P. B., Sommers H. M., Jennings R. B. (1965): A comparative study of the fine structure of normal and ischemic dog myocardium with special references to early changes following temporary occlusion of a coronary artery. Amer J. Pathol. **46,** 367—386

Hertz F., Cloarec A. (1984): Pharmacology of free radicals: recent views on their relation to inflamatory mechanism. Life Sci. **34,** 713—720

Hoffstein S., Weissmann G., Fox A. C. (1976): Lysosomes in myocardial infraction: Studies by means of cytochemistry and subcellular fractionation, with observations on the effects of methylprednisolone. Circulation **53,** 3 Suppl. I, I-34-I-40

Idell-Wenger J. A., Gryotohann L. W., Neely J. R. (1978): Coenzyme A and carnitine distribution in normal and ischemic hearts. J. Biol. Chem. **253,** 4310—4318

Ingwall J. S., DeLuca M., Sybers H. D., Wildenthal K. (1975): Fetal mouse hearts: A model for studying ischemia. Proc. Acad. Sci. USA **72,** 2809—2813

Jennings R. B., Ganote C. E. (1974): Structural changes in myocardium during acute ischemia. Circ. Res. (Suppl. III), **34,** III-156—168

Jennings R. B., Hawkins H. K. (1980): Ultrastructural changes of acute myocardial ischemia. In: Degradative Processes in Heart and Skeletal Muscle. (Ed. K. Wildenthal), pp. 295—346, Elsevier/North Holland Biochemical Press

Jennings R. B., Sommers H. M., Kaltenbach J. P., West J. J. (1964): Electrolyte alterations in acute myocardial ischemic injury. Circ. Res. **14,** 260—269

Jennings R. B., Hawkins H. K., Lowe J. E., Hill M. L., Klotman S., Reimer K. A. (1978): Relation between high energy phosphate and lethal injury in myocardial ischemia in the dog. Amer. J. Pathol. **92,** 187—214

Kane A. B., Young E. E., Schanne F. A. X., Farber J. L. (1980): Calcium dependence of phalloidin-induced liver cell death. Proc. Nat. Acad. Sci. USA **77,** 1171—1180

Kavanau J. L. (1963): Structure and function of biological membranes. Nature **198,** 525—530

Kloner R. A., Ganote C. E., Jennings R. B. (1974a): The "no-reflow" phenomenon after temporary coronary occlusion in the dog. J. Clin. Invest. **54,** 1496—1508

Kloner R. A., Ganote C. E., Whalen D., Jennings R. B. (1974b): Effect of a transient period of ischemia on myocardial cells. II. Fine structure during the first few minutes of reflow. Amer. J. Pathol. **74,** 399—420

McCallister L. P., Daiello D. C., Tyers G. F. O. (1978):Morphometric observations of the effects of normothermic ischemic area on dog myocardial ultrastructure. J. Mol. Cell. Cardiol. **10,** 67—80

Saito K., Kakei M., Uchimura S., Kashima T., Tanaka H. (1982): Toxic effects of chlorpromazine on red and white muscles in rat: and ultrastructural study. Toxicol. Appl. Pharmacol. **65,** 347—353

Schanne F. A. X., Kane A. B., Young E. E., Farber J. L. (1979): Calcium-dependence of toxic cell death: a final common pathway. Science **206,** 206—208

Schanne F. A. X., Pfau K. G., Farber J. L. (1980): Galactosamine-induced cell death in primary cultures of rat hepatocytes. Amer. J. Pathol. **100,** 25—36

Seeman P. (1972): The membrane actions of anesthetics and transquilizers. Pharmacol. Rev. **24,** 583—655

Shen A. C., Jennings R. B. (1972a): Myocardial calcium and magnesium in acute ischemic injury. Amer. J. Pathol. **67,** 417—440

Shen A. C., Jennings R. B. (1972b): Kinetics of calcium accumulation in acute myocardial ischemic injury. Amer. J. Pathol. **67,** 441—452

Shug A. L., Thomsen J. H., Folts J. D., Bittar N., Klein M. I., Koke J. R., Hutch P. J. (1978): Changes in tissue levels of carnitine and other metabolites during myocardial ischemia and anoxia. Arch. Biochim. Biophys. **187,** 25—33

Slezák J. (1969): Ultrastructural changes in early stages of experimentally induced myocardial infarction. Folia Morphol. **17,** 226—245

Slezák J., Tribulová N. (1975a): Morphological changes after simultaneous administration of isoproterenol (ISO) and K^+, Mg^{2+} — aspartate (KMg-ASP) as a physiological Ca^{2+} — antagonist. Arzneim. Forsch. Drug. Res. **25,** 918—923

Slezák J., Tribulová N. (1975b): Morphological changes after combined administration of isoproterenol and K^+, Mg^{2+} — aspartate as a physiological Ca^{2+} antagonist. In: Recent Advances in Studies on Cardiac Structure and Metabolism. Pathophysiology and Morphology of Myocardial Cell Alteration (Eds. A. Fleckenstein and G. Rona), V. 6, pp. 75—84, University Park Press, Baltimore

Slezák J., Tribulová N., Gabauer I., Holec V. (1982): Effect of chlorpromazine on postperfusion ischemical changes in myocardium. Bratisl. Lek. Listy **78,** 658—675

Slezák J., Geller S. A., Litwak R. S., Smith H., Jr. [1983a): Long-term study of the ultrastructural changes of myocardium in patients undergoing cardiac surgery, with more than 10 years follow-up. Internat. J. Cardiol. **4,** 153—168

Slezák J., Tribulová N., Gabauer I., Ziegelhöffer A., Holec V. (1983b): Manifestation of "oxygen paradox" in the myocardial sarcolemma and its prevention by chlorpromazine and protease inhibition. Conferences on Molecular Organization and Mechanisms of Membrane Function, Balatonfüred, September 19—23, pp. 128

Slezák J., Gabauer I., Ziegelhöffer A., Tribulová N., Styk J. (1984a): Salvage of reversibly ischemic myocardium by prevention of oxigen paradox. Effects of calmodulin and protease inhibitions. Quantitative evaluation. 5th Meeting of the European Section, International Society for Heart Research, Geneva, September 19—22

Slezák J., Tribulová N., Gabauer I., Ziegelhöffer A., Styk J. (1984b): Manifestation of oxygen paradox in ischemic reperfused myocardium and its prevention by chlorpromazine and protease inhibition. Bratisl. Lek. Listy **81,** 266—281

Šiška K., Ziegelhöffer A., Fedelešová M., Holec V., Slezák J., Styk J., Pancza D., Gabauer I. (1974): Effect of intra aortic balloon counterpulsation in experimental myocardial injury following acute coronary occlusion. Biochemical, ultrastructural and physiological aspects. Cardiovasc. Res. **8,** 404—414

Topping T. M., Travis D. F. (1974): An electron cytochemical study of mechanisms of lysosomal activity in the rat left ventricular mural myocardium. J. Ultrastruct. Res. **46,** 1—22

Toyo-oka T., Masaki T., Okamoto J., Tanak T. (1979): Calcium-activated neutral protease from bovine ventricular muscle: Isolation and some of its properties. J. Mol. Cell. Cardiol. **11,** 769—786

Van der Bosch H. (1974): Phosphoglyceride metabolism. Annu. Rev. Biochem. **43,** 243—277

Weglicki W. B., Waite M., Sisson P., Shohet S. B. (1971): Mitochondrial phospholipase A of microsomal and mitochondrial fraction. Biochim. Biophys. Acta **231,** 512—517

Welman E., Peters T. J. (1977a): Enhanced lysosome fragility in the anoxic perfused guinea pig heart: Effects of glucose and manitol. J. Mol. Cell. Cardiol. **9,** 101—120

Welman E., Peters T. J. (1977b): Prevention of lysosome distribution in anoxic myocardium by chloroquine and methyl prednisolone. Pharmacol. Res. Commun. **9,** 29—38

Whalen D. A., Jr., Hamilton D. G., Ganote C. E., Jennings R. B. (1974): Effect of a transient period a ischemia on myocardial cells. I. Effects on cell volume regulation. Amer. J. Pathol. **74,** 381—398

Whitmer J. T., Idell-Wenger J. A., Rovetto M. J., Neely J. R. (1978): Control of fatty acid metabolism in ischemic and hypoxic hearts. J. Biol. Chem. **253,** 4305—4309

Wildenthal K. (1975): Lysosomes and lysosomal enzymes in the heart. In: Lysosomes in Biology and Pathology. (Eds. J. T. Dingle and R. T. Dean), V. 4, pp 167—190, Amsterdam, North Holland Publishing

Ziegelhöffer A., Fedelešová M., Kvetňanský R. (1976): Myocardial catecholamines and related metabolic changes during acute coronary occlusion. In: Catecholamines and Stress (Eds. E. Usdin, R. Kvetňanský, I. J. Kopin), pp. 483—490, Pergamon Press, New York

Ziegelhöffer A., Das P. K., Sharma G. P., Singal P. K., Dhalla N. S. (1979): Propranolol effects on myocardial ultrastructure and high energy phosphates in anesthetized dogs subjected to ischemia and reperfusion. Can. J. Physiol. Pharmacol. **57,** 979—986

Zimmerman A. N. E., Hulsmann W. C. (1966): Paradoxical in influence of calcium ions on the permeability of the cell membranes of the isolated rat heart. Nature **211,** 646—647

Final version accepted April 8, 1987