

# A Study on the Mechanism of Myocardial Ischemic-Reperfusion Injury: The Role of Oxygen-free Radicals in Hypoxic-Reoxygenation Injury in Isolated Cardiomyocyte of Rat<sup>1</sup>

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**= Abstract =** The possible involvement of intracellularly-producing oxygen-free radicals in the genesis of the reperfusion injury of the heart was investigated in preparations of isolated cardiac myocyte. Calcium-tolerant cardiomyocytes were isolated from an adult rat heart by treatment with collagenase. The freshly isolated myocyte preparations contained more than 86% of viable cells by trypan blue exclusion criterion, with 84% or more of the cells showing the elongated rod-shaped morphology of the typical heart cell. The ATP content was 4.45  $\mu$  moles/g wet wt., which was comparable to that of the intact rat heart tissue. Incubation of the myocyte preparation in hypoxic medium with limited energy substrate and low calcium produced round-shaped cells associated with a decrease in number of the typical rod-shaped cells and total viable cells. Reoxygenation of the medium following a period of hypoxia incubation induced a further decrease in viable cell numbers with a concomitant increase in the release of intracellular enzymes (CPK, LDH) from the myocytes. The ATP content of the isolated cardiomyocyte rapidly decreased by hypoxic incubation, but this decrease was prevented considerably by reoxygenation. Digitonized cardiomyocyte preparations incubated with succinate produced a superoxide anion under aerobic conditions as well as under reoxygenated conditions following hypoxia. The capacity of superoxide anion production was much more highly maintained during the reoxygenation period than in the continuous aerobic condition. The cellular activities of defensive enzyme systems against oxygen free radicals, superoxide dismutase and glutathione peroxidase, were significantly lowered with hypoxic incubation. It is suggested from this study that during reoxygenation cardiomyocyte, and these radicals may contribute to the development of reoxygenation or reperfusion injury of the myocardium.

**Key words:** *Isolated cardiomyocyte, Reoxygenation injury, Oxygen-free radical*

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## INTRODUCTION

It has recently been suggested that reactive oxygen-free radicals play a certain role in the genesis of cellular damage in the reoxygenated hypoxic heart in vitro as well as in the reperfused ischemic myocardium in vivo. The involvement of oxygen-free radicals in the reoxygenation damage of myocardial tissue has been sup-

ported by observations that the exogenous scavengers of oxygen radicals and antioxidants prevent functional and biochemical derangements in isolated or in vivo ischemic-reperfused animal hearts (Kim and Akera 1987; Merrison *et al.* 1982; Shlafer *et al.* 1982; Stewart *et al.* 1983; Yoon *et al.* 1988).

Studies with isolated myocardial tissue indicate that ischemia causes an increase in catecholamine release, a disturbance of mitochondrial electron transport, an invasion of leukocytes, the conversion of xanthine dehydrogenase to xanthine oxidase and an accumulation of ATP breakdown products that act as substrates for oxygen radical production through the action of xanthine oxidase (Abrahamsson *et al.* 1984; Boveris and Chance 1973; Chambers *et al.* 1985; Lim and Kim 1988; Lucchesi and Mul-lane 1986). During reperfusion of ischemic tissue, all of the above conditions favor a burst of oxygen radical production (Fridovich 1979; Hess and Manson 1984; Meerson *et al.* 1982). In the previous studies using the isolated Langendorff preparations of rat hearts, we also measured an increased production of oxygen radicals upon reperfusion after a period of ischemia (Lee *et al.* 1987; Lim and Kim 1988).

In spite of this possible involvement of oxygen radicals in myocardial injury, there are still many debates about from where and by what mechanism oxygen radicals are produced in the ischemic-reperfused myocardium. Of the proposed sources and mechanisms, the extramyocardial cellular components—such as leukocytes invading the ischemic regions of the myocardium, the capillary endothelial xanthine oxidase system—have been the focus of many studies (Chambers *et al.* 1985; Lim and Kim 1988; Simpson and Lucchesi 1987). This may indicate that oxygen radicals produced from the extramyocardial sources play an important role in the genesis of myocardial cellular damage. Alternatively, however, there is a possibility that oxygen radicals from inside the myocardial cells are also involved directly in the cellular injury of a ischemic-reperfused heart. It has been reported that oxygen radicals are produced from the mitochondria and microsomal fractions of various tissues in in vitro conditions of anoxia-hyperoxia (Boveris and Chance 1973; Loschen and Azzi 1976; Turrens *et al.* 1982a & b). Currently, however,

definite evidence of intramyocardial production of oxygen radicals in in vivo heart and their role in the reperfusion injury of the ischemic myocardium is not available yet.

In the present study, we tried to perform the experiments with isolated cardiomyocytes to obtain more information about the intramyocardial production of oxygen radicals and their possible contributions to the genesis of reperfusion injury. Recent advances in cell isolation techniques provide the isolated cells with a number of experimental advantages in assessing the intracellular responses to the complex factors associated with many pathologic conditions. Preparations of the isolated cardiomyocytes can be incubated under controlled conditions free of neural, hemodynamic or other humoral influences in intact heart tissue. In addition, suspensions of the myocytes can be sampled serially so that the time course of cellular responses to the various vectors of damage associated with hypoxia and reoxygenation can be determined. In the present study, we isolated cardiac myocytes freshly from an adult rat heart and incubated them in in vitro conditions of hypoxia-reoxygenation simulating ischemia-reperfusion of an in vivo heart. During this incubation, the morphological and biochemical changes of the cells as well as the oxygen radical production in the myocytes were measured.

## MATERIALS AND METHODS

Sprague-Dawley rats of either sex, weighing 150–200 g were used. Adenosine diphosphate (ADP), adenosine monophosphate (AMP), antimycin A, t-butyl hydroperoxide, catalase, collagenase (Type I-A), creatine phosphate, creatinine, dimethylsulfoxide (DMSO), epinephrine, glucose-6-phosphate dehydrogenase, glutathione reductase, hexokinase, hyaluronidase (Type I-S), lactic dehydrogenase, myokinase, nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide phosphate (NADP), phosphoenol pyruvate, pyruvate kinase, reduced nicotinamide adenine dinucleotide (NADH), reduced nicotinamide adenine dinucleotide phosphate (NADPH), reduced glutathione (GSH), superoxide dismutase and taurine were purchased from the Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Digitonin was obtained from E. Merck (Darmstadt, West Germany), and other chemi-

als were reagent grade.

#### Isolation of cardiac myocytes

Calcium-tolerant cardiac myocytes were isolated freshly from rat hearts by modification of the collagenase method described by Farmer *et al.* (1983). Hearts of adult Sprague-Dawley rats (150–200 g), heparinized (100 IU, IP) 45 min before, were excised and perfused immediately by the retrograde perfusion mode of Langendorff apparatus. Preliminary perfusion was carried out for 15 min with a Krebs-Henseleit bicarbonate buffer solution (K-H solution) (in mM; NaCl 118, NaHCO<sub>3</sub> 27.2, KCl 4.8, MgSO<sub>4</sub>·7H<sub>2</sub>O 1.2, KH<sub>2</sub>PO<sub>4</sub> 1, CaCl<sub>2</sub> 1.25, glucose 11.1, pH 7.4 at 37°C) at a constant perfusion pressure of 100 cm H<sub>2</sub>O. After that, the hearts were perfused with nominally Ca<sup>++</sup>-free K-H solution at a flow rate of 10 ml/min for 4 min in order to wash out the Ca<sup>++</sup> from the interstitial tissues. Following this perfusion, the Ca<sup>++</sup>-free K-H solution supplemented with 0.05% collagenase (Sigma type I-A), 0.05% hyaluronidase, 0.1% bovine serum albumin (BSA) and 50 μM Ca<sup>++</sup> was perfused at a rate of 10 ml/min. After 5 min, the ventricles were removed from the perfusion apparatus by cutting at the atrio-ventricular junction. The ventricles, slashed twice vertically towards the apex, were placed in a 25 ml Erlenmeyer flask containing 5 ml of the same collagenase solution increased with a BSA concentration to 2%. Following a 5 min incubation with shaking (100 rev/min) at 37°C, the disaggregated myocytes were separated from the undigested tissue by sieving through a 300 μ nylon mesh into centrifuge tubes containing twice the volume of K-H solution with 50 μM Ca<sup>++</sup> and 2% BSA. The remaining undigested tissue was incubated for 2–3 more 5-min intervals in the same collagenase solution, and the separated myocytes were collected by filtration through nylon mesh in the same manner. The myocytes harvested and pooled from the digest were washed with a K-H solution with 50 μM Ca<sup>++</sup> and 2% BSA by centrifugation at 40 x g for 1 min. The myocyte pellet was washed three more times by suspending it in the solutions of which Ca<sup>++</sup> concentrations increased stepwise to 1 mM. All of the solutions used in the isolation procedure were saturated with a gas mixture of 95% O<sub>2</sub>-5% CO<sub>2</sub> and maintained at 37°C.

#### Experimental conditions for incubation of cardiac myocytes

The freshly isolated myocytes were further washed and incubated at 37°C in a K-H solution supplemented with 2% BSA, 3 mM taurine, 20 mM creatinine and with or without 1 mM Ca<sup>++</sup>. Myocytes were present at 500,000 cells/ml of the incubation solution. Aerobic incubations were carried out in a 25 ml Erlenmeyer flask under an atmosphere of 95% O<sub>2</sub>-5% CO<sub>2</sub>. Hypoxic incubations were performed in rubber-stoppered Erlenmeyer flasks gassed with a 95% N<sub>2</sub>-5% CO<sub>2</sub> mixture, and glucose was replaced by equimolar mannitol in the incubation medium. For observing the reoxygenation damage of the myocytes, hypoxic incubation conditions were changed into aerobic conditions by substituting 95% N<sub>2</sub>-5% CO<sub>2</sub> for 95% O<sub>2</sub>-5% CO<sub>2</sub>. Because of the fragility of the myocyte, the mechanical shaking was performed gently throughout the incubation.

#### Observations of the myocyte viability and injury

**Morphology:** Viability of the isolated myocyte was estimated using a 0.3% trypan blue exclusion test. The number of viable cells as well as the percentage of cells with the typical rod-shaped morphology of normal heart cells in situ and that of abnormally contracted round cells were calculated by using a hemocytometer and a light microscope (at X100–400). Prior to the trypan blue exclusion test and microscopy, the myocytes were slightly fixed with 1–2 drops of 2% glutaraldehyde to prevent the morphologic transformation of the cells during the procedures.

**Biochemical index:** The release of the intracellular enzymes, creatine phosphokinase (CPK) and lactic dehydrogenase (LDH), from the myocytes into the incubation medium, as well as, the cellular ATP content were measured for evaluating the degree of cellular injury during a period of various incubation conditions. Activities of CPK and LDH in the samples of the cell-free supernatant removed from the incubation mixture were analyzed by standard procedures using a UV-spectrophotometer as described by Bergmeyer (1985). For determination of the cellular content of ATP, an aliquot of myocyte suspension removed from the incubation mixture was centrifuged for 5 min at 300x g in a

refrigerated centrifuge. The cell pellet was deproteinized with 4 volumes of cold, 6% perchloric acid. In the neutralized perchloric acid extract, ATP was measured with standard enzymatic UV-methods (Bergmeyer 1985).

#### Measurement of superoxide anion production

The production of superoxide anion ( $O_2^-$ ) was estimated by measuring the superoxide dismutase (SOD)-inhibitable oxidation of epinephrine to adrenochrome (Misra and Fridovich 1972) in the digitonized myocytes. For digitonin lysis of the plasma membrane, freshly isolated myocytes were suspended in 4 volumes of cold (4°C) mannitol-sucrose medium containing 75 mg/ml digitonin, 230 mM mannitol, 70 mM sucrose, 1 mM EDTA and 5 mM Tris-HCl (pH 7.4). After centrifugation for 2 min at 1000x g, the sedimented pellet was washed three times with the mannitol-sucrose medium but without digitonin. The digitonized myocytes (2 mg protein/ml) were incubated at 37°C in the same mixture containing the mannitol-sucrose medium(-digitonin) supplemented with 1 mM epinephrine, 10 mM succinate, 150 U/ml catalase, 4  $\mu$ M antimycin A and with or without 150 U/ml SOD under hypoxic, aerobic or hypoxia following reoxygenation conditions. After the indicated periods of incubation, the formation of SOD-inhibitable adrenochrome was analyzed by using a dual-wavelength spectrophotometer (Aminco-Chance, U.S.A.) at a wavelength pair of 480/575 nm ( $\Sigma = 2,860/M/Cm$ ). The protein concentrations were determined by the method of Lowry *et al.* (1951).

#### Measurements of superoxide dismutase and glutathione peroxidase

Among intracellular defense systems against reactive oxygen radicals, superoxide dismutase and glutathione peroxidase (GSHPx), which scavenge  $O_2^-$  and  $H_2O_2$  respectively, were measured in the myocytes incubated under hypoxic conditions. After indicated time intervals, an aliquot of myocyte suspension was mixed with 3 volumes of 10 mM potassium phosphate-30 mM KCl solution (pH 7.4) and sonicated with an ultrasonic dismembrator (Quigley-Rochester Inc., U.S.A.) at maximum wattage for 10 sec, 10 times. After refrigerated centrifugation for 1 hr at 30,000x g, the enzyme activities were analyzed in the supernatant. SOD activity was measured

by the epinephrine auto-oxidation method of Misra and Fridovich (1972). An aliquot of the supernatant was added to the assay medium containing 50 mM  $NaHCO_3$ - $Na_2CO_3$  buffer (pH 10.2), 0.1 mM EDTA and 10 mM epinephrine. The rate of auto-oxidation of epinephrine to adrenochrome was monitored at 30°C by a UV-spectrophotometer (Perkin-Elmer, Model 139) at 325 nm. One unit of SOD is defined as the quantity of SOD required to produce a 50% inhibition of the rate of auto-oxidation of epinephrine under specified conditions. GSHPx activity was measured by the method of DeMaestro and McDonald (1985) using t-butylhydroperoxide as a substrate. The assay medium contained a 50 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 0.24 U/ml glutathione reductase, 1 mM GSH, 0.15 mM NADPH, 1.2 mM t-butylhydroperoxide and varying amounts of samples. The enzyme activity corresponding to the rate of disappearance of NADPH was monitored by a UV-spectrophotometer at 340 nm and 37°C. A unit of the enzyme is defined as the quantity of GSHPx required to consume 0.5  $\mu$ mole of NADPH per 1 min under specified conditions.

## RESULTS

### Properties of isolated cardiomyocytes

The freshly isolated myocyte preparations used in this study contained more than 86% of viable cells using the criterion of trypan blue exclusion with more than 84% of the cells showing the rod-shaped morphology of typical normal heart cells (Fig. 1, Table 1). The ATP content of these cells was  $4.45 \pm 0.43$   $\mu$ mole/g.wet wt (fig. 2). This value was comparable to that of intact rat heart tissue and to the reported values by other researchers (Farmer *et al.* 1983; Hohl *et al.* 1982; Kao *et al.* 1980). When the myocytes were incubated aerobically in the presence of glucose, there was only a small decline in the number of viable cells at a rate of less than 5%/hr. This did not differ regardless of the presence or absence of  $Ca^{++}$  (1 mM) in the incubation medium (Fig. 2, Table 1). The ATP content was not so markedly changed for up to 2 hr of aerobic incubation with glucose (Fig. 2). When the myocytes were incubated anaerobically in the absence of glucose, there was a marked decrease in the number of viable, rod-

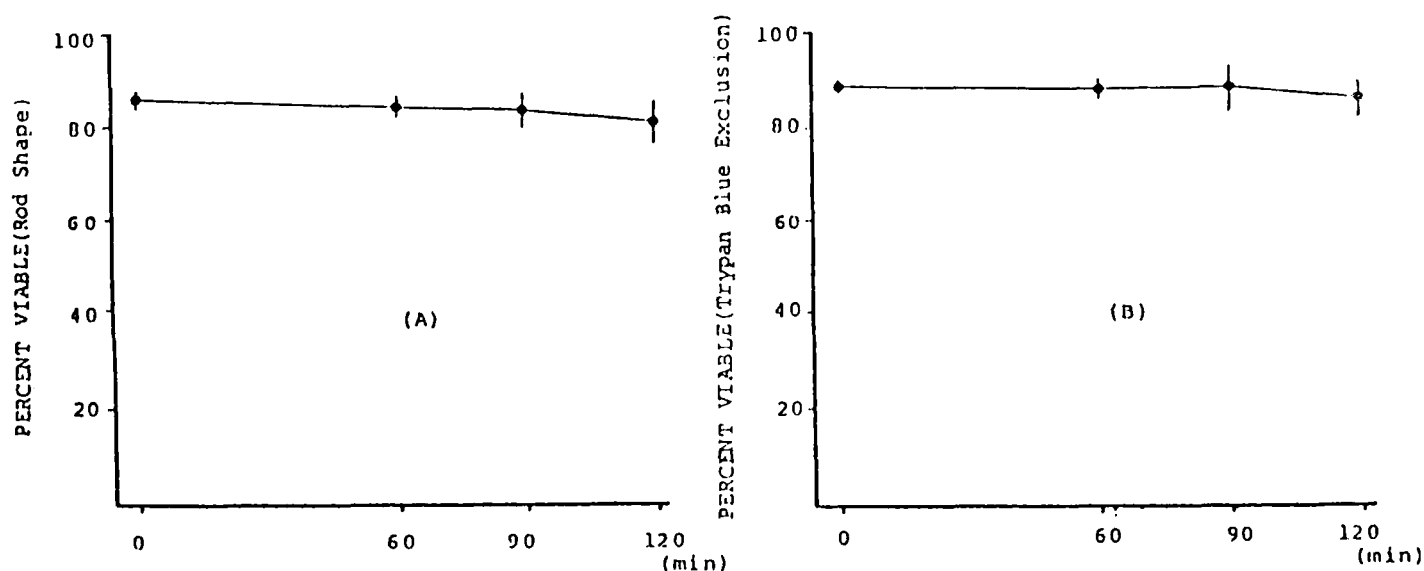


Fig. 1. Retention of viability of cardiomyocytes isolated from adult rat heart. Isolated cardiomyocytes ( $5 \times 10^5$ /ml) were incubated at 37°C in calcium-free Krebs-Henseleit solution supplemented with 2% bovine serum albumin, 30 mM taurine and 20 mM creatinine under aerobic conditions gassed with 95% O<sub>2</sub>-5% CO<sub>2</sub> mixture. Panel(A) represents percent of viable cells maintaining rod-shaped morphology, and panel(B) percent of cells able to exclude trypan blue(0.3%). Mean  $\pm$  S.E. of 6 experiments.

Table 1. Viability and morphology of isolated cardiomyocytes of adult rat under various incubation conditions

	Incubation Conditions		Viable*(%)			Non-Viable(%)		
	Ca <sup>++</sup> (1 mM)	Glucose (10 mM)	Rod	Round	Total	Rod	Round	Total
Control	not incubated		84.5 $\pm$ 3.1	2.2 $\pm$ 1.2	86.7	0	13.3 $\pm$ 1.9	13.3
Aerobic 60 min	+	+	79.0 $\pm$ 5.0	3.0 $\pm$ 1.0	82	0	18.0 $\pm$ 5.0	18.0
"    90 min	-	+	83.3 $\pm$ 6.2	3.8 $\pm$ 1.5	87.1	0	12.9 $\pm$ 4.6	12.9
Anaerobic 60 min	+	-	1***	13***	14***	0	86	86
"    90 min	-	-	50.8*** $\pm$ 15.4	5.6*** $\pm$ 2.1	56.4***	9.4 $\pm$ 4.7	34.2 $\pm$ 9.1	43.6
Anaerobic 45 min	+	-	1***	0***	1***	0	99	99
Aerobic 15 min								
Anaerobic 60 min	-	+**	15.6***	29.4***	45***	8.4	46.6	55
Aerobic 30 min			$\pm$ 3.4	$\pm$ 7.9		$\pm$ 3.4	$\pm$ 8.1	

\*: Viability was determined using the criterion of 0.3% trypan blue exclusion.

Mean  $\pm$  S.E. of 6 experiments.

\*\* : Glucose was added during reoxygenation period only.

\*\*\* : P < 0.01, vs Control by t-test.

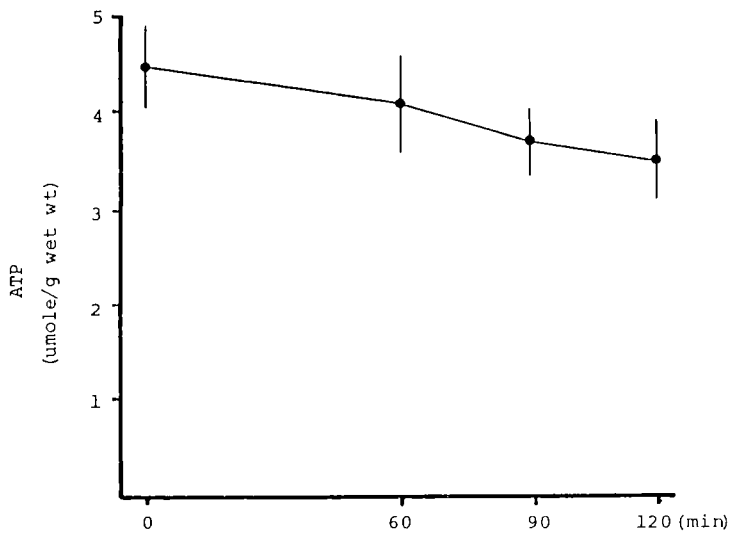


Fig. 2. ATP content of isolated cardiomyocyte of adult rat incubated under aerobic conditions. Incubation conditions are same as in Fig. 1.

-shaped cells associated with an increase in the number of non-viable, round-shaped contracted cells. These changes were accelerated by the addition of 1 mM  $Ca^{++}$  to the incubation medium (Table 1).

#### Responses of isolated cardiomyocytes to the hypoxic-reoxygenation transition

**Viability:** Hypoxic incubation (atmosphere of 95%  $N_2$ -5%  $CO_2$ ) of the myocytes in the absence of glucose and  $Ca^{++}$  produced a decrease in number of viable, rod-shaped cells at a rate of about 20%/hr. This loss of viability of typical rod-shaped cells was accelerated by reoxygenation of the incubation medium by gassing 95%  $O_2$ -5%  $CO_2$  mixture (Fig. 3-A). On the other hand, the number of viable cells excluding trypan blue but showing atypical round-shaped

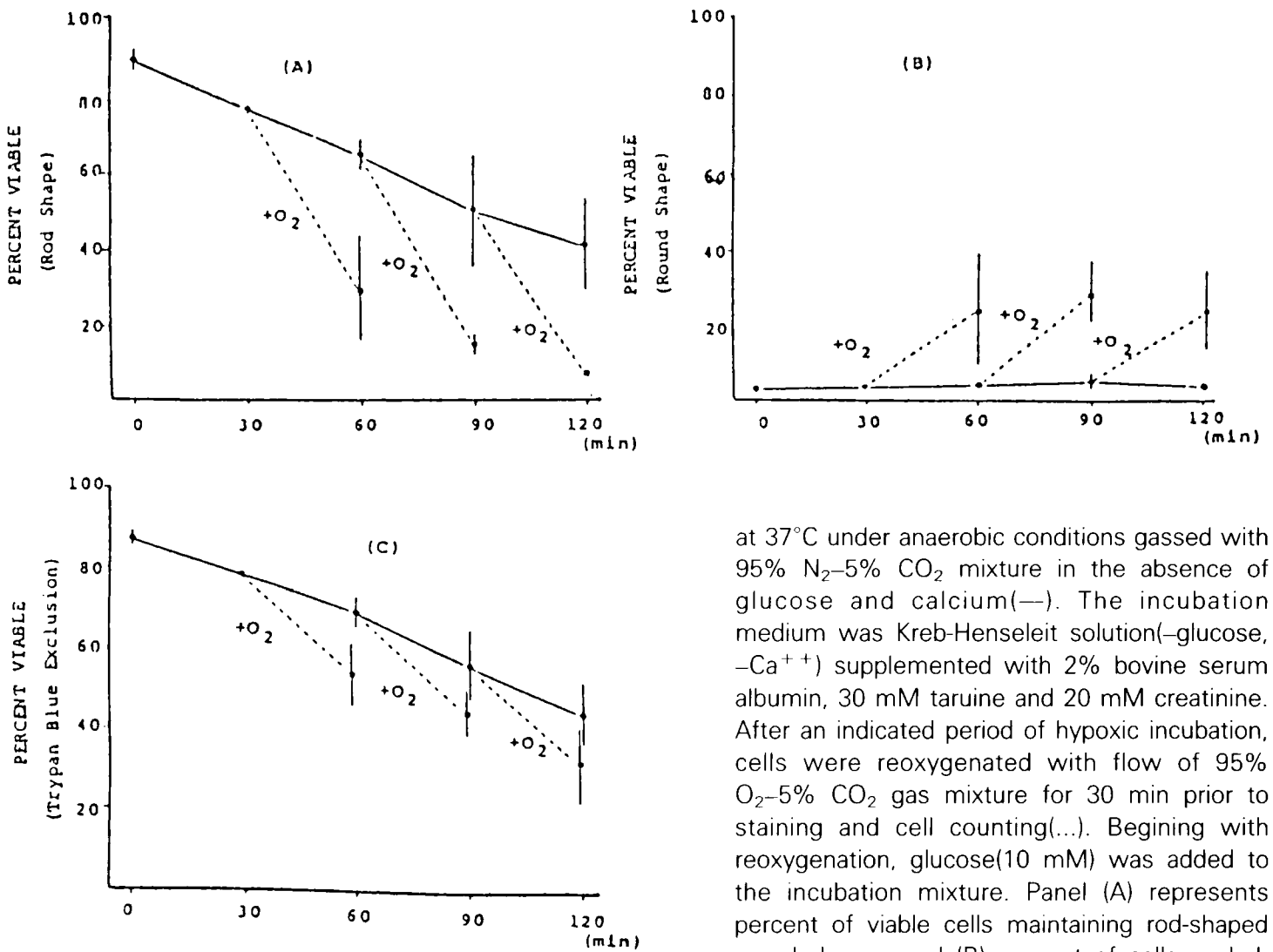


Fig. 3. Influence of hypoxic to reoxygenation transition on viability of isolated cardiomyocytes of adult rat. Isolated cells ( $5 \times 10^5$ /ml) were incubated

at 37°C under anaerobic conditions gassed with 95%  $N_2$ -5%  $CO_2$  mixture in the absence of glucose and calcium(—). The incubation medium was Krebs-Henseleit solution(-glucose, - $Ca^{++}$ ) supplemented with 2% bovine serum albumin, 30 mM taruine and 20 mM creatinine. After an indicated period of hypoxic incubation, cells were reoxygenated with flow of 95%  $O_2$ -5%  $CO_2$  gas mixture for 30 min prior to staining and cell counting(...). Beginning with reoxygenation, glucose(10 mM) was added to the incubation mixture. Panel (A) represents percent of viable cells maintaining rod-shaped morphology, panel (B) percent of cells excluding trypan blue(0.3%) but maintaining round-shaped morphology, and panel (C) percent of total viable cells excluding trypan blue. Mean  $\pm$  S.E. of 6 experiments.

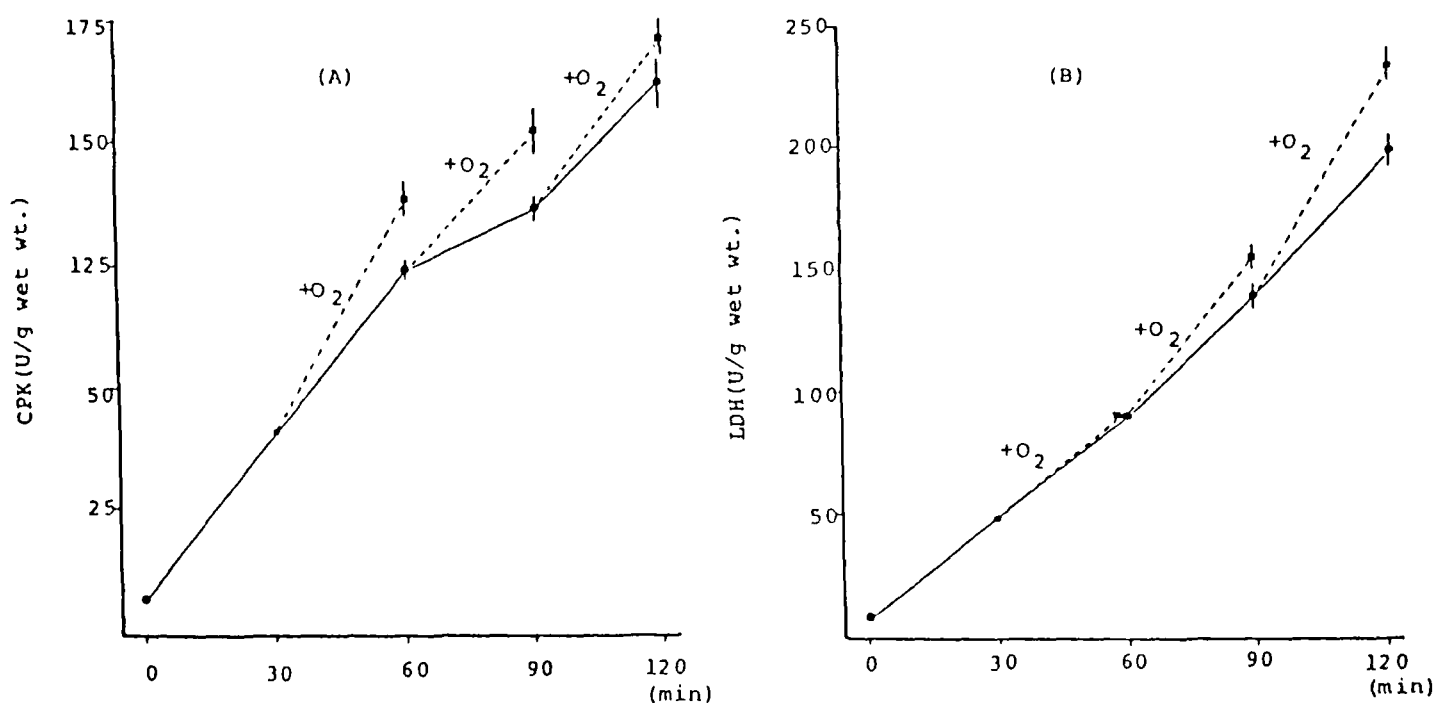


Fig. 4. Influence of hypoxic to reoxygenation transition on release of intracellular enzymes from isolated cardiomyocytes of adult rat heart. Hypoxic and reoxygenated incubation conditions are same as in Fig. 3. Panel (A) represents CPK release, and panel (B) LDH release. Mean  $\pm$  S.E. of 7 experiments.

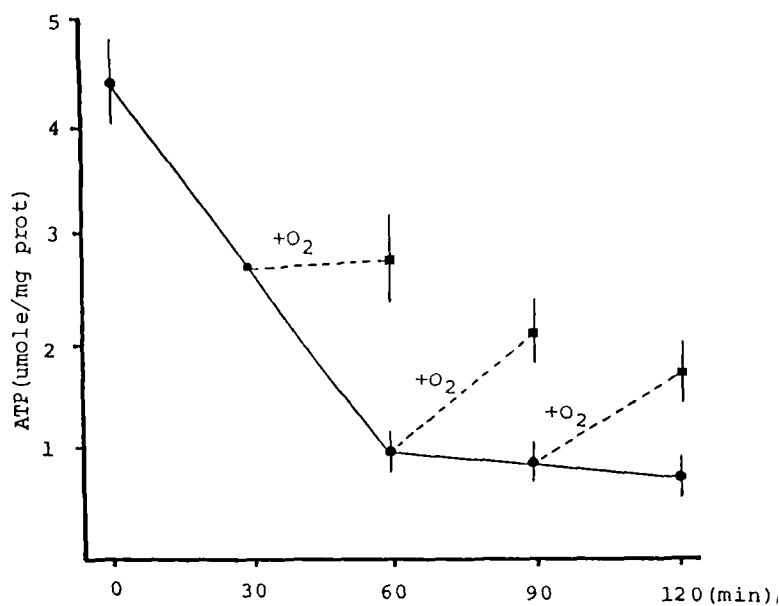


Fig. 5. Influence of hypoxic to reoxygenation transition on ATP content of isolated cardiomyocytes of adult rat heart. Hypoxic and reoxygenated conditions are same as in Fig. 3. Mean  $\pm$  S.E. of 6 experiments.

morphology, which was less than 5% of the total cell numbers in the hypoxic condition, was increased by reoxygenation (Fig. 3-B). However, the total number of viable cells excluding trypan blue, regardless of the morphology, was further decreased by hypoxic to reoxygenation transition

of the incubation condition (Fig. 3-C).

**Release of intracellular enzymes:** Release of CPK and LDH from the myocytes incubated in the hypoxic medium without glucose and  $Ca^{++}$  was markedly increased with a lapse of the incubation period. This increase was accelerated by reoxygenation of the incubation medium (Fig. 4).

**ATP content:** The myocytes incubated under hypoxic conditions in the absence of glucose and  $Ca^{++}$  lost ATP precipitously showing less than 20% of the normal content within 1 hr of incubation. Reoxygenation of these hypoxic cells, however, resulted in a recovery of ATP content to a considerable extent but not to the normal value (Fig. 5).

#### Effect of oxygen radical scavengers on the hypoxic-reoxygenated injury of the cardiomyocytes

As noted above, the hypoxic to reoxygenation transition of the incubation condition produced injurious results to the isolated cardiomyocytes. This was considered as an *in vitro* expression of the ischemic-reperfusion injury seen in the *in vivo* hearts. Since oxygen radicals are thought to be involved in the *in vivo* reperfusion injury, the protective effects of several oxygen radical

**Table 2.** Effects of oxygen radical scavengers on viability and intracellular enzyme release in hypoxic-reoxygenated isolated cardiomyocytes of adult rat

Conditions	Viable cell(%)*			CPK (U/g wet wt.)	LDH (U/g wet wt.)
	Rod	Round	Total		
Control	84.5±3.1	2.2±1.2	86.7	7.4± 1.3	5.1± 2.0
Anaerobic 60 min <sup>1</sup> + Aerobic 30 min	15.6±1.4	29.4±7.9	45	103.1±27.0	154.7± 2.2
+SOD <sup>2</sup> & Cat <sup>3</sup>	14.6±4.4	26.5±7.7	41.1	106.2±28.8	145.8±28.6
+DMSO <sup>4</sup>	30.6±6.5**	14.8±4.7**	50.8	104.7±25.2	145.8±31.2

\*: Determined by 0.3% trypan blue exclusion. Mean ± S.E. of 6 experiments.

\*\* : P < 0.01. 1 vs 4 by t-test

2: Superoxide dismutase, 0.5 mg/ml

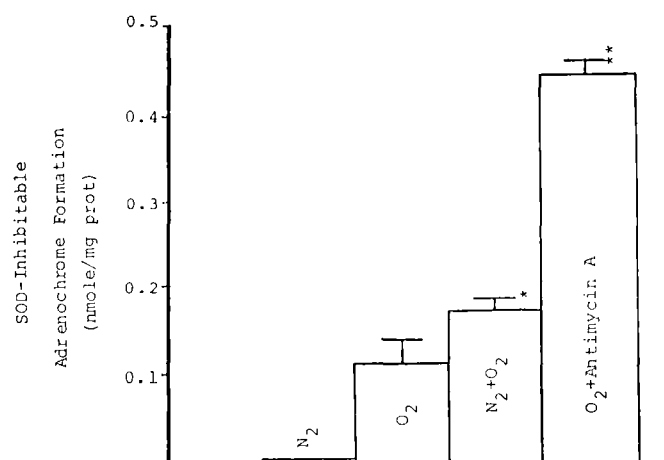
3: Catalase, 0.5 mg/ml

4: Dimethylsulfoxide, 5%

scavengers (SOD, catalase, DMSO) to the hypoxic-reoxygenation injury of the isolated cardiomyocytes were also examined. Unexpectedly, however, all the scavengers studied showed no significant protective effect either on the loss of cell viability or on the increased release of intracellular enzymes during the hypoxic to reoxygenation transition of the incubation. Only DMSO (5%) slightly prevented from a decrease in the number of viable rod cells (Table 2).

**Superoxide anion production from isolated cardiomyocytes**

Superoxide anion production from the digitonized cardiomyocytes incubated with succinate was estimated by measuring the SOD-inhibitable oxidation of epinephrine to adrenochrome. When the digitonized myocytes were incubated in aerobic conditions, superoxide anion was produced in a considerable amount. After 30 min under aerobic conditions, the SOD-inhibitable adrenochrome formation was 0.115 ± 0.027 nmole/mg.prot. Reoxygenation (for 30 min) of the myocytes after 1 hr of hypoxic incubation resulted in a further increase in adrenochrome formation (0.171 ± 0.017 nmole/mg.prot). In the case of aerobic incubation of the myocyte with antimycin A, the formation of SOD-inhibitable adrenochrome was much higher than that without the mitochondrial respiratory inhibition (Fig. 6). In the hypoxic conditions, the superoxide anion was not produced.



**Fig. 6.** Superoxide anion production from digitonized cardiomyocytes of adult rat in hypoxic, aerobic and reoxygenation conditions. Isolated cells were treated with low level of digitonin as described in Method. Digitonin-lysed cells(2 mg prot/ml) were incubated at 37°C in mannitol-sucrose media supplemented with succinate (10 mM), catalase(150 U/ml) and epinephrine(1 mM) under the presence or the absence of SOD(150 U/ml). Formation of SOD-inhibitable adrenochrome from epinephrine during 30 min of incubation under the hypoxic(95% N<sub>2</sub>-5% CO<sub>2</sub>) and aerobic(95% O<sub>2</sub>-5% CO<sub>2</sub>) conditions, and during 60 min of hypoxia followed by 30 min of reoxygenation was measured with dual-wavelength spectrophotometer at a wavelength pair of 480/575 nm. In one incubation under aerobic condition, antimycin A(4 uM) was added. Mean±S.E. of 6 experiments. \*: P<0.05. against aerobic incubation. \*\*: P<0.01. against aerobic incubation by t-test.



**Table 3.** Influence of hypoxia on superoxide dismutase and glutathione peroxidase activities of isolated cells from adult rat heart\*

Conditions	SOD (U/g. wet. wt)	GSHPx (U/g. wet. wt)
Control	2667.7 ± 233.2	20.0 ± 2.7
Anaerobic 1 hr	2555.0 ± 217.6	13.2 ± 3.2***
2 hr	1925.6 ± 140.7**	5.0 ± 1.9***
3 hr	1509.1 ± 122.3**	2.8 ± 1.4***

\*: Mean ± S.E. of 6 experiments

\*\* : P < 0.01. superoxid dismutase, vs control by t-test

\*\*\*: P < 0.01. glutathione peroxidase, vs control by t-test

#### Defensive enzymes against oxygen radicals in hypoxic cardiomyocytes

In the freshly isolated cardiomyocytes the activity of superoxide dismutase was  $2,667 \pm 233$  U/g wet wt. When the myocytes were incubated hypoxically in the absence of glucose, the enzyme activity declined gradually, showing 56% of that of the fresh cell at 3 hr of incubation. Glutathion peroxidase activity was  $20.0 \pm 2.7$  U/g wet wt in the freshly isolated myocytes. After a 3-hr incubation under hypoxic conditions, the enzyme activity decreased to  $2.8 \pm 1.4$  U/g wet wt (Table 3).

#### DISCUSSION

In past studies using isolated cardiomyocyte preparations, the intolerability of the cells to  $Ca^{++}$  was the most difficult problem in conducting the experiment. However, recent advances in isolation techniques have made it possible to prepare myocytes tolerant to physiologic concentrations of  $Ca^{++}$  (Farmer *et al.* 1983). We also isolated  $Ca^{++}$ -tolerant cardiomyocytes from adult rat hearts. The fresh myocytes that were incubated in aerobic conditions retained their normal properties for a long time regardless of the presence or absence of  $Ca^{++}$  (1 mM). In hypoxic incubation conditions without energy substrates, however, the myocytes deteriorated more easily in the presence of  $Ca^{++}$  than without  $Ca^{++}$  (Table 1). This was thought to be the result of energy depletion, in which case the passive influx of  $Ca^{++}$  from the extra- and intra-

cellular stores may increase and promote cellular deterioration.

In the present study, the extent of cellular injuries, in terms of morphologic changes and intracellular enzyme release, seen in the hypoxically incubated cardiomyocytes was accelerated upon reoxygenation with the 95%  $O_2$ -5%  $CO_2$  gas mixture. This was thought to be analogous to the reperfusion injury of ischemic myocardium in in vivo hearts and was indicative of a probable involvement of reactive oxygen radicals in the hypoxic-reoxygenated injury of the isolated cardiomyocyte. This possible involvement of oxygen radical was also supported by the increased production of superoxide anion in the digitonized myocytes incubated under the condition of hypoxia followed by reoxygenation (Fig. 6). However, in disaccordance with this, this hypoxic-reoxygenated injury was not prevented by the oxygen radical scavenging enzymes, SOD and catalase. This apparent failure of the protective effect may be accounted for by the inaccessibility of the high molecular weight enzymes to the intracellular sites of oxygen-radical production. Since oxygen radicals are highly reactive and exist for a very short time, the radicals can not be eliminated effectively by the scavengers unless they are located very closely in the radical production sites (Freeman and Crapo 1982).

In normal mitochondria, proximity to 5% of the electron flow results in superoxide anion production at the NADH dehydrogenase step and near to the ubiquinone component (Boveris and Chance 1973; Forman and boveris 1982; Loschen and Azzi 1976). In normal situations, this superoxide anion is eliminated by the endogenously-existing superoxide dismutase in mitochondria as well as in the cytosole so as not to produce any harmful effects to the cell. In the hypoxic-reoxygenated cells, however, it is likely that mitochondrial functions are well altered to produce more superoxide anion resulting in cellular injury. It is thought that the disturbances of mitochondrial electron transport and the resulting accumulation of reducing equivalents during hypoxic conditions lead to increased productions of oxygen radicals when previously hypoxic cells are reoxygenated (Hess and Manson 1984). In in vitro studies with extracted mitochondria, the production of superoxide anions has been reported to increase in hypoxic or in hyperoxic

incubation conditions (Leyck and Parnham 1988; Turrens *et al.* 1982a & b). Currently, however, because of the methodological difficulties for detecting the production of oxygen radicals in *in vivo* situations, it is not clear whether mitochondria act as a major source of oxygen radicals in ischemic-reperfused hearts.

Low concentrations of digitonin lyse the plasma membrane and increase the membrane permeability of isolated cardiomyocytes without alterations to the mitochondrial structures and functions (Altschuld *et al.* 1981; Murphy *et al.* 1982). In the present study, the digitonized myocytes incubated aerobically with succinate as a mitochondrial respiratory substrate produced superoxide anion. This superoxide production was increased in the mitochondria of which respiration had been inhibited by antimycin A as well as in the incubation conditions of reoxygenation following a period of hypoxia (Fig. 6). These results indicate that oxygen radicals can be produced intracellularly in larger amount in the hypoxic-reoxygenated cells of which mitochondrial functions are depressed. These are in accordance with reports that oxygen consumption at state 4 respiration is much higher in the damaged mitochondria than in the intact mitochondria (Boveris and Chance 1973; Loschen *et al.* 1971).

In spite of increased oxygen radical production, if cellular defense systems against oxygen radicals are intact, the radicals may not exert any deleterious effects upon the cells. Studies with mitochondria extracted from ischemic tissues indicated the depressed activities in the oxygen radical scavenging enzymes (Majewska *et al.* 1978; Schlafer *et al.* 1987). In the present study, the activities of superoxide dismutase and glutathione peroxidase were also observed to decrease in the hypoxically incubated myocytes. This suggests that the reduction of intracellular defense systems against oxygen radicals may also contribute to the development of the oxygen radical-linked reoxygenation injury of the cardiomyocytes.

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= 국문초록 =

## 허혈심근 재관류 손상의 기전에 관한 연구 : 흰쥐심근 분리세포의 저산소-산소재공급손상에 있어서 산소라디칼의 역할

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허혈심근 재관류 손상시 심근세포내 출처로부터의 산소라디칼 생성과 이의 심근손상에 대한 기여를 규명하기 위한 목적으로 분리심근세포에서 저산소에 이은 산소재공급시 세포손상을 관찰하고 산소라디칼의 생성을 측정하였다. 성숙 흰쥐 심실근에서 collagenase를 이용한 방법으로 칼슘-내성 심근세포를 분리하였다. 신선분리심근세포는 trypan blue에 염색되지 않는 생존세포가 총세포의 86% 이상을 차지하였고, 전형적 심근세포모양인 막대형의 정상생존세포는 84% 이상을 차지하였으며, ATP 함량은  $4.45 \mu\text{mole/g wet wt}$ 로 정상 심근조직과 유사하였다. Glucose 및 칼슘이 존재하지 않는 저산소의 혐기성 조건에서는 trypan blue에 염색되지 않으나 형태학적으로는 구형을 보이는 비전형 생존세포가 증가하였지만, 정형의 막대형 세포 및 총생존 세포수는 감소하였다. 저산소에 이은 산소재공급은 생존세포의 더욱 현저한 감소를 나타내었으며, 이와 병행하여 세포질 효소인 CPK 및 LDH 유리도 산소재공급시 현저히 증가하였다. 저산소 조건에서 ATP 함량은 급격히 감소하였으나 산소재공급에 의하여 상당량이 회복되었다. Antimycin A 및/또는 succinate 존재하에서 digitonin 처리세포는 호기성조건에서 뿐 아니라 저산소후 산소재공급시에도 superoxide anion을 생성하였으며, 특히 호기성 조건보다 저산소에 이은 산소재공급시 생성물이 더욱 높았다. 산소라디칼에 대한 방어효소인 superoxide dismutase 및 glutathione peroxidase 활성도는 저산소 조건에서 현저히 감소되었다. 이상의 결과에서 허혈-재관류 또는 저산소-산소 재공급시 심근세포 자체내에서도 산소라디칼이 생성되고 세포손상에 참여할 가능성이 있을 것으로 사료되었다.