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NEUTROPHILS-INDUCED INCREASE OF ADENOSINE TRIPHOSPHATE DEPLETION IN RAT NEONATAL CARDIAC MYOCYTES WITH IMPAIRED ENERGY METABOLISM.

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Isolated, cultured rat neonatal cardiac myocytes were placed in medium suppled mented with mitochondrial respiratory inhibitor potassium cyanide which caused a rapid adenosine triphosphate (ATP) depletion. These myocytes with the impaired energy metabolism (*"hypoxia-like state"*) were exposed to unstimulated human neutrophils. Effect of human neutrophils on the myocytes in the *"hypoxia-like state"* was quantified as a total change in the amount of ATP in cardiac cells. After 5 hours of incubation of neutrophils with the myocytes in the *"hypoxia-like state"* an additional decrease (of 50 per cent) in ATP content was observed. Since catalase (which destroys hydrogen peroxide) prevented the further decline in ATP level in the myocytes with impaired energy metabolism, it seem that hydrogen peroxide and possibly their products are responsible for this effect. These results suggest that unstimulated human neutrophils after activation by the contact with injured cardiac cells caused further decrease of ATP level in target cells.

Key words: *neutrophilis, adenosine triphosphate, cardiac myocytes.*

INTRODUCTION

Neutrophils infiltrating ischemic myocardium may release a variety of factors capable to mediate the injury of tissue. First, membrane bound NADPH oxidase transforms molecular oxygen to superoxide anion followed by a secondary production of other reactive oxygen intermediates (ROI) such as hydroxyl radical (·OH) and hydrogen peroxide (1). Second, activation of phospholipase A2 leads to the release of arachidonic acid (AA) metabolites (2), and finally, activated neutrophils release a variety of proteolytic enzymes from their specific and azurophilic granules (3). All these neutrophils-derived mediatots may participate in provoking cellular injury in tissues inflitrated

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by these cells. That leukocytes are involved in the inflammatory process after acute myocardial infarct is known, but whether ROI, proteolytic enzymes or AA metabolites are produced by neutrophils in this situation remain uncertain (4). In our experimental system unstimulated effector cells (neutrophils) were incubated with metabolically injured target cells i. e., rat neonatal cardiac myocytes obtained from primary monolayers culture, and treated with potassium cyanide causing "*hypoxia-like state*". We regard this system as reflecting the situation *in vivo*, in which circulating "*resting*" neutrophils are being activated by ischemic myocardium and hypothesize that the injured myocytes would lead to the activation of resting neutrophils which, in turn, would further contribute to the myocyte damage.

MATERIALS AND METHODS

Media and chemicals.

Minimal essential medium (MEM) and phosphate-buffered saline (PBS) were obtained from Biomed, Poland; foetal calf serum (FCS) from Flow Laboratories, Rockville, USA; and ATP Monitoring Reagent from LBK-Wallac, Turku, Finland. Catalase (CAT), superoxide dismutase (SOD), and all other reagents were purchased from Sigma Corp., St. Louis, USA.

Isolation of myocytes.

Cardiac myocytes were isolated from 1 to 3 days-old Wistar rats according to the methods of Harrary and Farley (5) and Halle and Wollenberger [6]; details of the procedure were described earlier (7, 8). Cells were resuspended in MEM enriched with l-glutamine (1 mM) and 10% inactivated FCS (culture medium) and cultured at 37° C, 5% CO_2 and 100% humidity for 72 hours. The cells were then scraped from the surface of the dishes with a rubber policeman and counted.

Treatment of myocytes with potassium cyanide.

Myocytes were incubated in the medium supplemented with the potassium cyanide ($7{,}5 \times 10^{-4}$ M) a convenient inducer of hypoxia in biological system, due to its potent inhibition of cytochrome oxidase, the predominant O_2-consuming enzyme required for mitochondrial ATP production. After 4 hours the myocytes in the "*hypoxia-like state*" were used to further experiments with neutrophils. Control samples were maintained in growth medium without the inhibitor.

Isolation of neutrophils.

Neutrophils were obtained from heparinized venous blood of healthy volunteers by the Gradisol G (Polfa, Warsaw) gradient centrifugation. Residual erythrocytes were lysed by hypotonic shock. Cell viability as assessed by the trypan blue exclusion always exceeded 95 per cent. The cell suspension was washed three times with PBS, resuspended in the culture medium and used immediately.

Neutrophils — myocytes interaction bioassay.

One x10^6 unstimulated neutrophils (effectors) were seeded onto wells containing 1×10^5 target myocytes in the "*hypoxia-like state*", suspended in the medium with potassium cyanide ($7{,}5 \times 10^{-4}$ M). The medium was supplemented with 1 mM glucose. It has been repeatedly shown that ATP is produced in neutrophils principally via the glycolytic pathway (9, 10) and that the mitochondrial inhibitors like potassium cyanide have no effect on the ATP level in neutrophils both in the presence or absence of glucose (9). The intracellular content of ATP in human neutrophils was found to be 4.5 ± 0.2 fmol per cell (9). This level remained constant during 5 hours of incubation in the culture medium containing potassium cyanide (data not shown). As the intracellular content of ATP in rat neonatal cardiac myocytes was found to be 10^{-11} M (8, 11), the ATP level in neutrophils at the neutrophils-to-myocytes ratio 10 : 1 could be neglected. The final volume of the reaction mixture was adjusted to 500 μl with the culture medium. After incubation for 1 to 5 hours at 37 C in the humidified atmsphere with 5% CO_2 and 95% O_2, 20 μl of the cell suspension was removed to determine the content of ATP.

ATP measurement.

Intracellular ATP level was determined according to the firefly luciferase luminometric method (11). Briefly, the cells were incubated up to 5 hr at 37° C, 100% humidity and 5% CO_2. An aliquot of Triton X-100 was added (final concentration-5%) to disintegrate the cell membrane and to release the intracellular ATP. Twenty μl samples were taken at time zero and at different intervals thereafter and transferred to the cuvettes containing 800 μl of Tris-EDTA buffer. After 60-second incubation at room temperature 200 μl of the luciferin-luciferase enzyme (LKB 1243-200 ATP Monitoring Reagent) was added and the ATP level measured. The protein was determined by Lowry method (12).

RESULTS

Intracellular ATP content after 4 hours of incubation of rat neonatal cardiac myocytes in the medium containing $7{,}5 \times 10^{-4}$ M potassium cyanide decreased by 54% *(Fig. 1 Panel A)*. The cells with impaired energy metabolism were then incubated with unstimulated human neutrophils. The exposure of these myocytes to neutrophils resulted in a further 50 percent drop in total ATP level after 5 hours of incubation *(Fig. 1 Panel B)*. As judged by trypan blue exclussion $>98\%$ of the cardiac cells were viable after 5 hours incubation with neutrophils. Since, as indicated in Materials and Methods, in our experimental system the ATP content of neutrophils could be neglected this drop indicated the decrease in ATP content of the "*hypoxia-like myocytes*". In control cardiac myocytes incubated with KCN no differences in ATP level were observed within 4—9 hours (data not shown).

The contact of primarily unstimulated neutrophils (i. e., incubated in the absence of any artificial stimulator) with the hypoxic myocytes resulted in the further drop in the ATP content of myocytes. For this induced by the hypoxic myocytes state of neutrophils we use the term 'activation'. If the

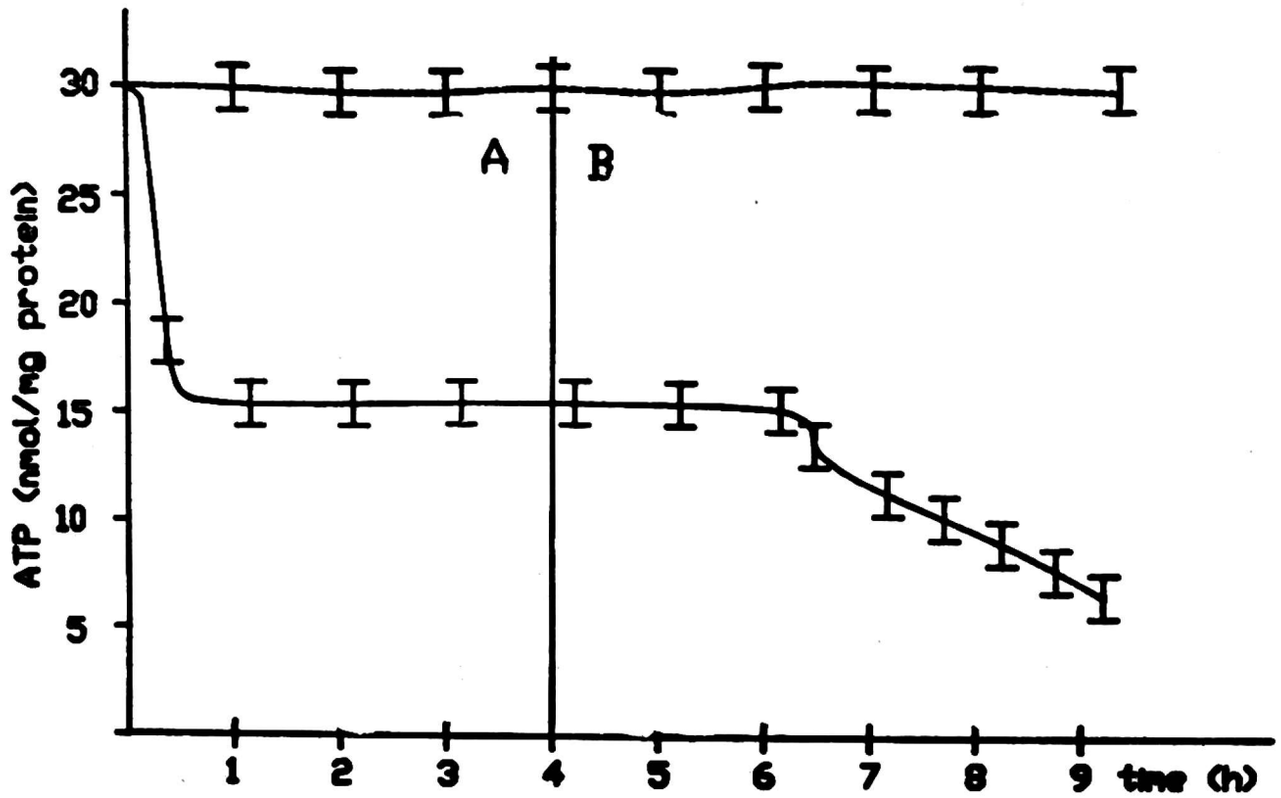

Fig. 1. Time-course of the human neutrophils-induced ATP depletion in rat neonatal cardiac myod cytes. Rat myocytes (1×10^5/ml) were incubated in the MEM medium (supplemented with 1 mM glucose) in presence (—) or absence (---) of 7.5×10^{-4}M potassium cyanide (Panel A). After 4 hours of incubation the myocytes with mitochondrial respiratory inhibitor, unstimulated human neutrophils (1×10^6/ml) were added and co-incubated for the next 5 hours (Panel B). Results are expressed as mean ± 1 SD od eight experiments.

healthy myocytes had any other activatory effect on the neutrophils it was not connected with the following changes in ATP content of cardiac cells.

In control experiments (cultures of myocytes without KCN co-incubated with unstimulated neutrophils), no change in total ATP was observed, indicating that neutrophils were not activated under these conditions *(Fig. 1 Panel B)*. To explain the participation of reactive oxygen intermediates (ROI) in this phenomenon, the exogenous scavengers of ROI were added.

The neutrophils-induced ATP depletion in cardiac cells was efficiently prevented by catalase alone and by the mixture of catalase and superoxide dismutase but not by superoxide dismutase alone *(Fig. 2)*. These enzymes were ineffective when added to the cell mixture incubation with a delay of 3—5 hours *(Fig. 3)*. Heat-inactivated catalase was also ineffective (data not shown).

The effect of Nonsteroidal Antiinflammatory drugs (NSAID) on the neutrophils-mediated decrease in ATP level in target cells is shown in *Fig. 4*. Cycloxygenase inhibitors indomethacin, ibuprofen and naproxen, as well as the cyclo-and lipoxygenase inhibitor, timegadine used in concentration 10^{-5} M had none or only minor protective effect on ATP level in "*hypoxia-like*" myocytes. Lower concentrations of these drugs (i. e., 10^{-6}—10^{-7} M) were also inneffective in this respect (data not shown).

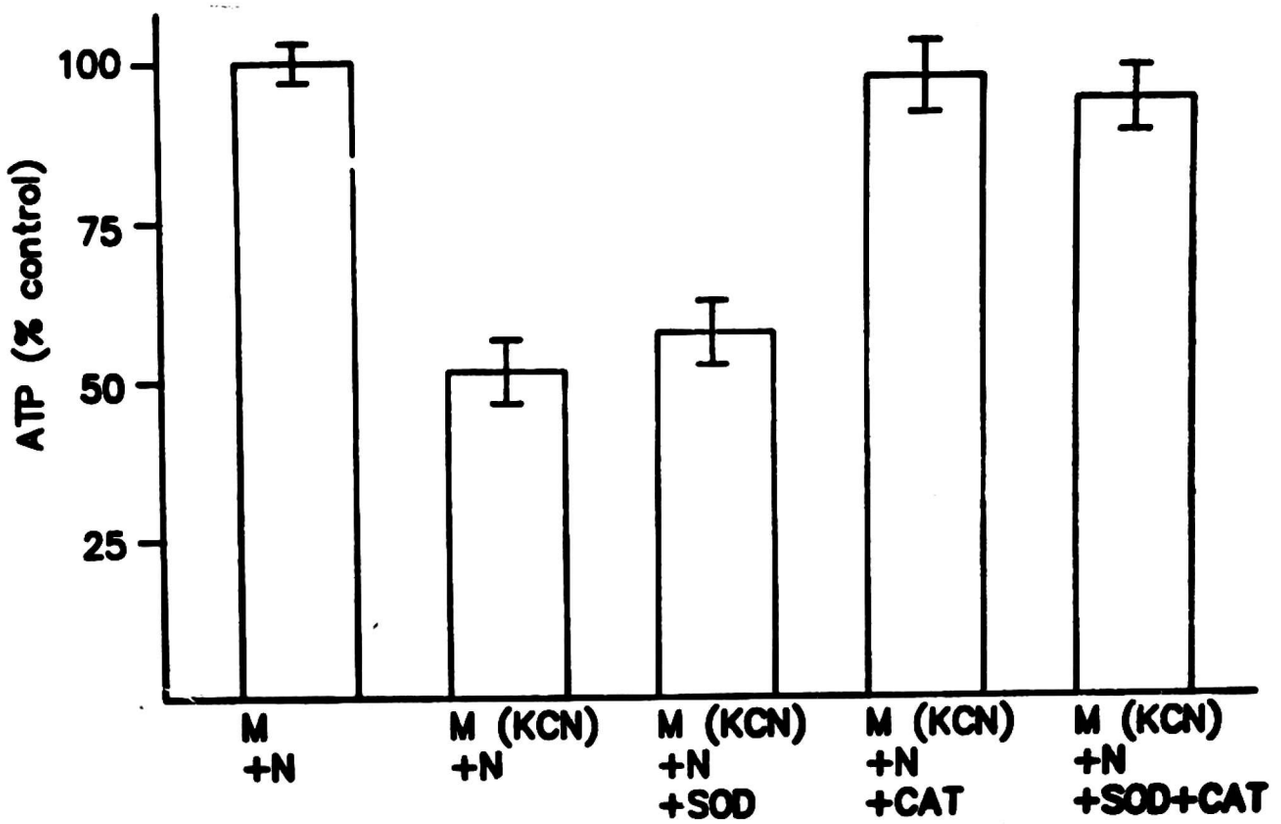

Fig. 2. The effects of scavengers of reactive oxygen intermediates (ROI) on ATP level in rat neo natal cardiac myocytes [M]. The myocytes were incubated in the medium containing 7.5×10^{-4}M KCN. After 4 hours unstimulated human neutrophils [N] (1×10^6/ml) were added to the myocytes (1×10^5/ml) and co-incubated for the next 5 hours. Scavengers were added at the same time as neutrophils. Superoxide Dismutase (SOD) = 300 U; Catalase (CAT) = 4000 U. Results are expressed as mean ±1 SD of nine experiments.

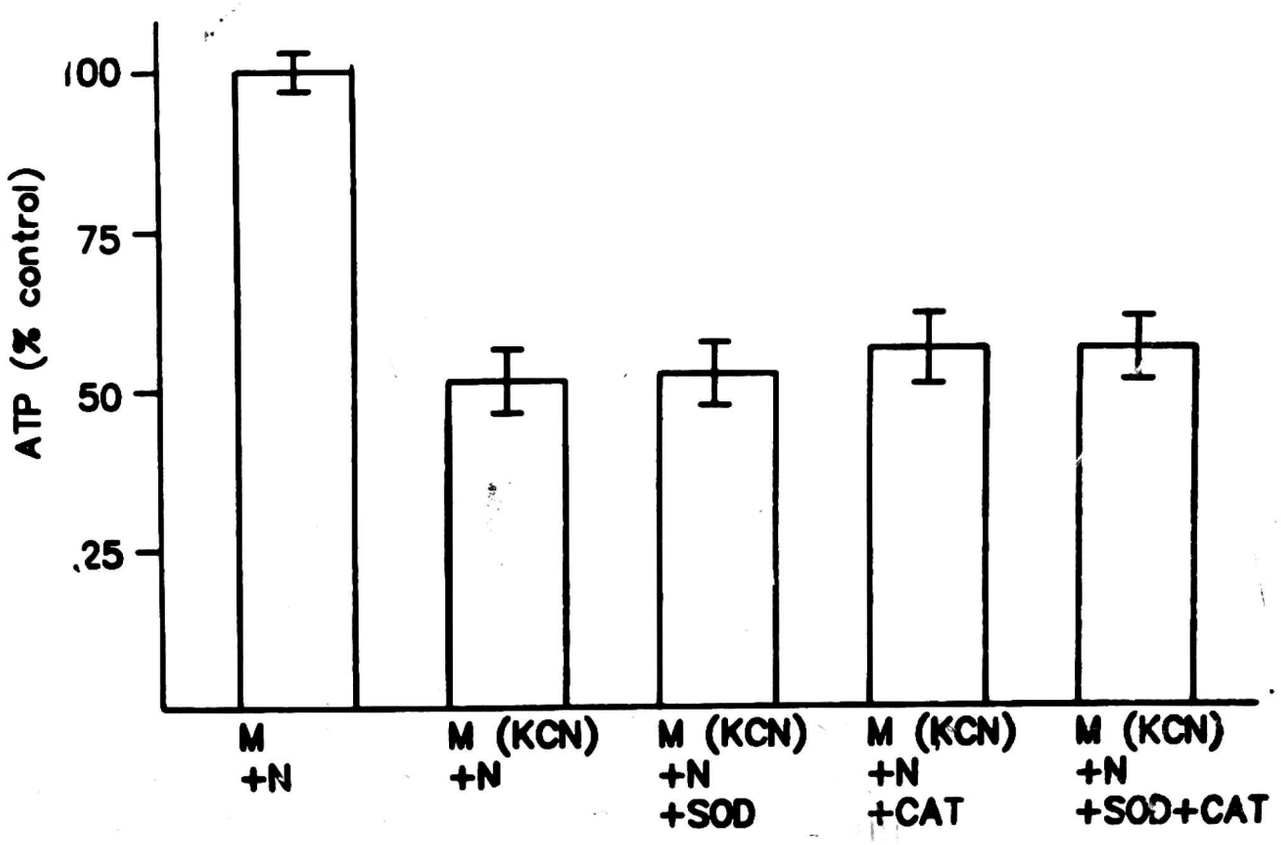

Fig. 3. The effect of scavengers of ROI on ATP level in rat cardiac myocytes. After 4 hours incubation the myocytes with 7.5×10^{-4}M KCN human unstimulated neutrophils were added (in ratio 10 : 1) and co-incubated for the 3 hours. After this time the scavengers of ROI (SOD = 300 U, CAT = 4000 U) were added and co-incubated for the next 2 hours. Results are expressed as mean ±1 SD of nine experiments.

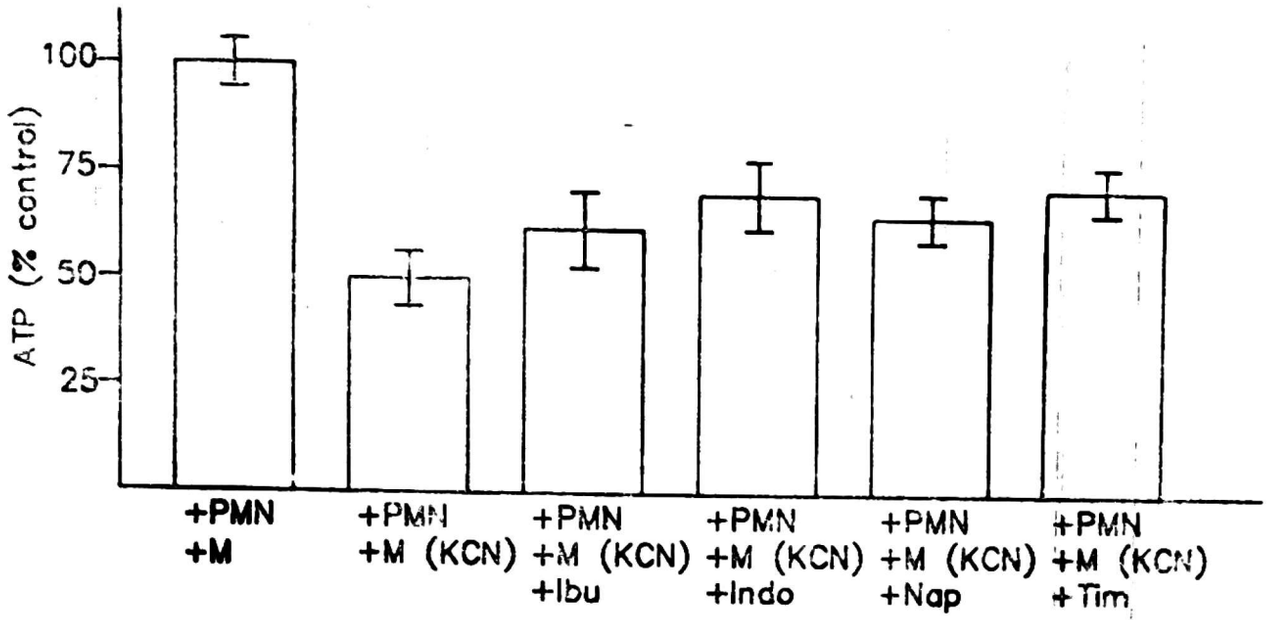

Fig. 4. Effect of Nonsteroidal Antiinflammatory Drugs (NSAID) on ATP level in rat neonatal cardiac myocytes (M). Myocytes were incubated in the medium containing 7.5×10^{-4} M KCN [M(KCN)] or in the medium without KCN [M]. After 4 hours unstimulated neutrophils (1×10^4/ml) were added to the myocytes (1×10^5/ml) and co-incubated for the next 5 hours in the presence or absence of 10^{-5} M of NSAID (Ibuprofen-Ibu.; Indomethacin-Indo.; Naproxen-Nap.; Timegadine--Tim.). Results are expressed as mean ± 1 SD of nine experiments.

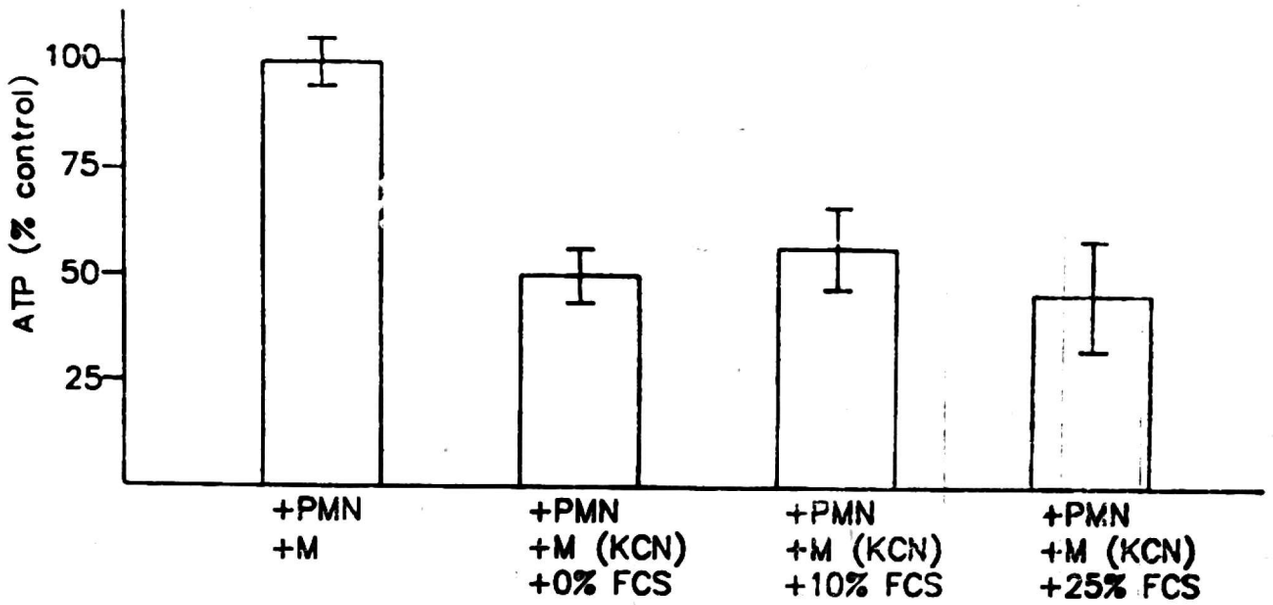

Fig. 5. Effect of foetal calf serum (FCS) on ATP level in rat myocytes [M]. After 4 hours incubation the myocytes with 7.5×10^{-4} M KCN human unstimulated neutrophils were added (in ratio 10 : 1) and co-incubated for the next 5 hours in the presence (10%, 25%) or absence (0%) of FCS. Results are expressed as mean ± 1 SD of nine experiments.

To test the role of proteinases possibly released by neutrophils activated by the contact with injured myocytes, the effector and target cells were co-incubated in the presence of foetal calf serum (FCS), containing naturally occuring antiproteases. *Fig. 5* shows that FCS in any of the used concentrations did not protect the ATP level in "*hypoxic-like*" myocytes exposed to neutrophils.

DISCUSSION

The possible involvement of neutrophils in the mechanism(s) of post-ischemic damage of the heart is an important issue in the pathophysiology of myocardial injury. Neutrophils may initiate a cascade of reactions that leads to the production of the ROI, proteolytic enzymes or metabolites of arachidonic acid (1, 2, 3), but the mechanisms by which intravascular neutrophils cause the injury of ischemic myocardium is not completely understood (4).

In experimental systems generally accepted as the "*in vitro*" models of the processes operating at the sites of inflammation, neutrophils are activated with artificial stimuli and incubated with intact target cells. We regard these systems inappropriate, because the mechanism of cytotoxic effect of neutrophils (ROI, proteolytic enzymes or metabolites of AA) is dependent on the stimulus used (2, 13).

In our experiments unstimulated neutrophils were exposed to cardiac myocytes with the impaired energy metabolism. Since the ischemic myocardium produces certain amount of high-energy phosphate by the anaerobic glycolysis pathway and the tissue acidosis develops rapidly the myocytes were incubated only with potassium cyanide (mitochondrial respiratory inhibitor) without blocking the anaerobic glycolysis. On the other hand, it has been found that ATP is produced in neutrophils via the glycolytic pathway (9, 10) thus the mitochondrial inhibitors have no effect on the energy status of neutrophils.

It should be noted that in the present study human neutrophils were used as effectors against rat neonatal cardiac myocytes. However, the attack of neutrophils on target cells does not depend on the recognition of major histocompatibility antigens (MHC-unrestricted); thus it was not necessary to use neutrophils and myocytes originating from inbred animals or from the same species (14).

We shown that after 3 hours of co-incubation the neutrophils were activated by the contact with injured myocytes leading to the further depletion in ATP level in the target cells. The observed delay of 3 hours depended mainly on the basic contect of ATP in the damaged myocytes (data not shown).

Many sources of activation of unstimulated neutrophils by the injured myocytes may be proposed. It was recently demonstrated (15) that myocytes previously injured by the metabolic inhibitors release the platelet activating factor (PAF). PAF is widely regarded as the proinflammatory inducer of platelets and neutrophils aggregation and degranulation. Dreyer et. al. (16) provided a direct evidence supporting the hypothesis that canine neutrophils could be activated by cardiac lymph obtained during reperfusion of ischemic myocardium. These results demonstrate the possibility of activation of neu-

trophils by ischemic myocardium but the mechanism by which neutrophils affect this tissue damage remaines uncertain. In our experimental system hydrogen peroxide seemes to play a major role in the mechanism of injuring of cardiac neonatal myocytes by neutrophils. We have shown that catalase (hydrogen peroxide decomposing enzyme) or a combination of catalase and superoxide dismutase added to myocytes-neutrophils mixture significantly protected the ATP level in this culture. The absence of protection of the ATP level by scavengers of ROI added after 3 hours to neutrophils being incubated with myocytes could be explained by the existence of intimate contact between these cells. It was recently shown (17) that intercellular adhesion of canine neutrophils to intact cardiac myocytes from adult was low and unchanged by stimulation of the neutrophils with opsonized zymosan. The intercellular adhesion significantly increased only when both myocytes and neutrophils were stimulated and it was shown that production of hydrogen peroxide was associated with this adhesion.

It seems that this close contact promote the formation of microenvironment at the interface between the neutrophils and myocytes, where the concentration of hydrogen peroxide, released by neutrophils, may occur and escape the protective actions of scavengers.

On the other hand, the cycloxygenase inhibitors indomethacin, ibuprofen and naproxen, as well as the cyclo- and lipoxygenase inhibitor, timegadine had none or little protective effect on the ATP level in "*hypoxia-like*" myocytes exposed to neutrophils.

Also proteinase-mediated mechanism of activity of neutrophils under these conditions seems unlikely since FCS, containing naturally occuring antiproteinases, used in concentration in which inhibited by 70% the toxic effect of PMA-stimulated neutrophils on rat hepatocytes (18) had no protective effect on ATP level in myocytes in our experimental system.

In these studies we address the hypothesis that "*hypoxia-like state*" in cultured myocytes promotes the activation of unstimulated neutrophils against the injured target cells. The results demonstrate that human neutrophils activated by contact with injured cardiac myocytes release hydrogen peroxide which causes further depletion of ATP level in the "*hypoxia-like state*" myocytes. It is suggested that similar activation may be also responsible for the injury of ischemic myocardium by the circulating neutrophils *in vivo*.

REFERENCES

1. Kukreja RC, Weaver AB, Hess ML. Stimulated human neutrophils damage cardiac sarcoplasmic reticulum function by generation of oxidants. Biochim Biophys Acta 1989; 198: 990—995.

2. Bentwood BJ. Henson PM. The sequential release of granule constituents from human neutrophils. *J Immunol* 1980; 855; 124—134.
3. Weissmann G, Smolen JE, Korchak HM. Release of inflammatory mediators from stimulated neutrophils. *N Engl J Med* 1980; 27: 303—305.
4. Semb AG, Vaage J, Mjos OP. Oxygen free radical producing leukocytes cause functional depression of isolated rat hearts; role of Leukotrienes. *J Mol Cell Cardiol* 1990; 22: 555—563.
5. Harrary J. Farley B. In vitro studies on single beating rat heart cells. I. Growth and organization. *Exp Cell Res* 1963; 29: 451—464.
6. Halle W, Wollenberger A. Die Differenzierung isolieter Herzzellen in einem chemisch definierten Nährmedium. *Ztschr Zellforsch* 1968; 87: 292—305.
7. Grąbczewska E, Laskowska-Bożek H, Maśliński S, Ryżewski J. Cultured beating myocytes from neonatal rat heart as a model for the study of muscarinic cholinergic receptors. *Int J Tiss. React* 1983; 5: 165—171.
8. Gajewski M, Laskowska-Bożek, H, Orlewski P, Maśliński S, Ryżewski J. Influence of lipid peroxidation and hydrogen peroxide on muscarinic cholinergic receptors and ATP level in rat myocytes and lymphocytes. *Int J Tiss React* 1988; 5: 281—291.
9. Lane TA, Lamkin GE. A reassessment of the energy requirements for neutrophil migration. Adenosine Triphosphate depletion enhances chemotaxis. *Blood* 1984; 64; 986—993.
10. Borregaard N, Herlin T. Energy metabolism of human neutrophils during phagocytosis. *J Clin Invest* 1982; 70: 550—557.
11. Noronha Dutra AA, Steen EM. Lipid peroxidation as a mechanism of injury in cardiac myocytes. *Lab Invest* 1982; 47: 346—356.
12. Lowry OH, Rosebrough NJ, Farr Al, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; 193: 265—275.
13. Test ST, Weiss SJ. Quantitative and temporal characterization of the extracellular H_2O_2 pool generated by human neutrophils. *J Biol Chem* 1984; 399: 259—263.
14. Heffel DF, Costa AK, Schieble TM, Truddel JR. Neutrophil mediated cytotoxicity in confluent monolayers of rat hepatocytes. *Biochem Arch* 1989; 5: 229—235.
15. Janero DR, Burghardt C. Production and release of platelet-activating factor by the injured heart-muscle cells (cardiomyocytes). *Res Commun Chem Pathol Pharmacol* 1990; 67: 201—218.
16. Dreyer WJ, Smith CW, Michael LH et al. Canine neutrophil activation by cardiac lymph obtained during reperfusion by ischemic myocardium. *Circ Res* 1989: 65: 1751—1762.
17. Entman ML, Youker K, Shappel SB et al. Neutrophils adherence to isolated adult canine myocytes. Evidence for a CD 18-dependent mechanism. *J Clin Invest* 1990; 85: 1497—1506.
18. Guigui B, Rosenbaum J, Preaux A, et al. Toxicity of phorbol myristate acetate-stimulated polymorphonuclear neutrophils against rat hepatocytes. Demonstration and mechanism. *Lab Invest* 1988; 59: 831—843.

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