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# The role of mitogen-activated protein kinases in hydrogen peroxide-induced myogenic cell apoptosis

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Transplantation of autologous skeletal muscle-derived stem cells into injured heart may limit scar thinning and dilatation and improve myocardial function. Success of cellular cardiomyoplasty depends on the integration and survival of the engrafted cells in injured myocardial tissue. A great part of transplanted cells die in the first hours after transplantation as a consequence of activation and release of various bioactive substances such as reactive oxygen species (ROS) and cytokines in the damaged heart tissue. The purpose of this work was to elucidate the role of mitogen-activated kinases (MAPK) in ROS-induced myogenic cell apoptosis. Adult rabbit skeletal muscle stem cells were exposed to increasing concentrations of $H_2O_2$. We found that apoptotic concentrations of $H_2O_2$ transiently activated JNK and p38 MAP kinases, whereas the effect of adding $H_2O_2$ on ERK phosphorylation was negligible. Specific inhibition of ERK, JNK and p38 MAPK increased the apoptotic effect of 500–1000 μM $H_2O_2$. These results suggest the protective role of ERK, JNK and p38 MAP kinases in oxidative stress-induced myogenic cell apoptosis.

**Key words:** myogenic cells, MAP kinase, apoptosis

## INTRODUCTION

The transplantation of different types of progenitor or stem cells into diseased hearts, known as cellular cardiomyoplasty, is a new promising concept to treat heart insufficiency and myocardial infarction. Success of cellular cardiomyoplasty depends on the integration and survival of the engrafted cells in the injured myocardial tissue. Various kinds of stress may affect the transplanted cells in the damaged cardiac tissue; therefore, the viability of transplanted donor myoblasts must be considered. Heart diseases, such as myocardial infarction, cause loss of cardiomyocyte as well as transplanted cells [1, 2]. Activation and release of various bioactive substances such as reactive oxygen species (ROS) and cytokines are responsible for the induction of myocardial apoptosis. ROS have a well-established role as physiological modulators of skeletal muscle functions, ranging from development to metabolism and from blood flow to contractile functions. Moreover, ROS may contribute to the development of muscle fatigue, inflammation, and degeneration, and may be implicated in many muscular diseases as well as in the stimulation of either hypertrophy or apoptosis in cardiac myocytes [3, 4]. But the role of ROS originated in the injured heart in the death and survival of the transplanted myogenic cells is still not clear.

Mitogen-activated protein kinases (MAPKs) function in cellular signal transduction cascades and are activated by a diverse range of stimuli including heart infarct followed by ischemia, and bioactive agents. Three major mitogen-activated protein kinase families were identified as the extracellular signal-regulated kinases (ERK), c-Jun $NH_2$-terminal protein kinases (JNK), and p38 MAP kinases. Activation of MAPK family plays the key role in the pathogenesis of various processes in the heart, e. g., myocardial hypertrophy and its transition to heart failure, in ischemic and reperfusion injury, as well in the cardioprotection conferred by ischemia- or pharmacologically induced preconditioning [5]. It has been established that $H_2O_2$-induced cardiac myocyte apoptosis is associated with a dynamic change in expression genes whose products potentially influence the progression of apoptotic response. Despite these extensive investigations, the role of ERK, JNK, and p38 MAP kinases in muscle derived stem cells seems contradictory.

Recently, we have established primary cell lines from rabbit muscle, proved their myogenic origin by detection of desmin as well as the potential to differentiate into skeletal myosin positive cells and showed that these myogenic cells could be maintained in tissue culture for many months without any decline in their proliferation [6]. The purpose of this study was to determine the role of individual MAP kinases in hydrogen peroxide ($H_2O_2$) induced myogenic cell apoptosis. Our results indicated a transient activation of JNK and p38 MAPK after exposure to apoptotic concentrations of $H_2O$. The use of specific ERK, JNK and p38 MAPK inhibitors suggested a protective role of these signaling molecules in primary myogenic cells after $H_2O_2$ treatment.

## MATERIALS AND METHODS

**Isolation and cultivation of myogenic cells.** Primary myogenic cells were isolated from rabbit skeletal muscle under sterile conditions and general rabbit anaesthesia. A pierce of skeletal

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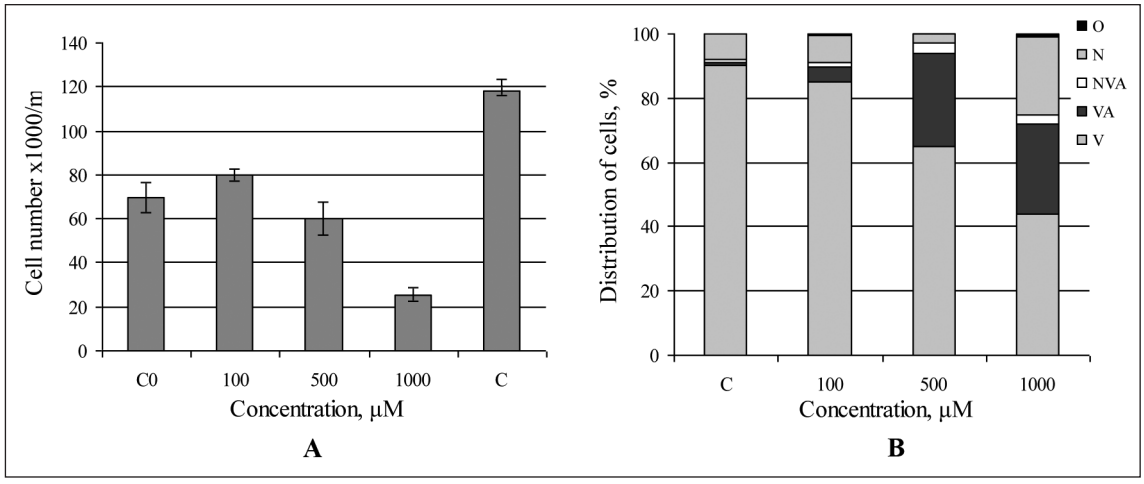


**Fig. 1.** Dose-dependent decrease in viable cell number (A) and distribution of cells in the population after a 20 h exposure to $H_2O_2$, according to the staining with AO/EB as described in Materials and Methods. V – viable, VA – viable apoptotic, NVA – non-viable apoptotic, N – necrotic cells, O – cells without chromatin

muscle tissue (0.3 $cm^3$) was minced and exposed to a digestive solution containing 0.125% of trypsin-EDTA (Sigma), 1 mg/ml collagenase and 0.3 mg/ml hyaluronidase (Biological Industries) in PBS as described in [7]. The tube was incubated for 15–45 min at 37 °C in a shaker bath. The cell mass was washed with Iscove's modified Dulbeco medium (IDMEM) (Sigma), supplemented with fetal calf serum (FCS) (10%), penicillin (100 U/ml) and streptomycin (100 μg/ml) (Biological Industries). The amount of cells was counted using a haematocytometer, and cell viability was evaluated by the trypan blue exclusion test. Cells were maintained at 37 °C in humidified atmospheric air with 5% of $CO_2$ and passaged twice a week detaching cells with 0.25% (w/v) trypsin / EDTA solution (Biological industries).

The licence for the use of laboratory animals in stem cell research (No. 0121, 2004-07-09) was received from the Lithuanian Food and Veterinary office.

**Cell viability and apoptosis assay.** Cells were exposed for 20 h to 100, 500 and 1000 μM of $H_2O_2$. Cell viability was expressed as a number of viable cells counted using a haematocytometer and trypan blue dye. Fluorescent microscopy was used to determine the mode of cell death by using two fluorescent dyes, acridine orange and ethidium bromide (AO/EB). Cells were categorized as follows: viable, viable apoptotic, non-viable apoptotic, necrotic and others [8]. The experiments were repeated 3–5 times.

**Immunoblot analysis.** After appropriate treatments, the cells were rinsed and solubilized in an ice-cold lysis buffer. Equal amounts of protein were separated by SDS-PAGE on 10% acrylamide gels and transferred to Roti®-PVDF membranes. Subsequently, the membranes were incubated in a blocking buffer, probed with the primary antibody to phospho-ERK, phospho-JNK1/2, phospho-p38 (Cell Signaling Technology) followed by horseradish peroxidase-conjugated secondary antibodies. Protein bands were detected with an enhanced chemiluminescence system according to manufacturer's instructions (Pierce Biotech).

**Statistical analysis.** Significance tests were performed using the statistical package of MS Excel 2003 program. Data represent the means of at least three independent experiments. They are expressed as mean ± standard deviations (SD).

## RESULTS AND DISCUSSION

Recently it has been shown that low concentrations of $H_2O_2$ increased protein synthesis without affecting the survival of adult rat ventricular myocytes, whereas higher concentrations of $H_2O_2$ caused both apoptosis and necrosis [4, 9]. To elucidate whether $H_2O_2$ induces apoptosis in muscle-derived adult stem cells, myogenic cells isolated from rabbit skeletal muscle were exposed to increasing concentrations of $H_2O_2$ for 20 h. The treatment with 100 μM $H_2O_2$ resulted in an inhibition of cell proliferation, whereas higher concentrations induced apoptotic cell death (Fig. 1). The part of necrotic cells increased from 3 ± 1% after treatment with 500 μM $H_2O_2$ to 26 ± 3% after treatment with 1000 μM $H_2O_2$. Activation of caspases is a hallmark of apoptosis in most biological systems. We detected caspase-3 cleavage in myogenic cells after exposure to 500 μM $H_2O_2$ (Fig. 2).

ROS may initiate cell death processes through affecting various signaling cascades, including activation of MAPKs. $H_2O_2$ has been shown to activate MAP kinases in a number of cell types as well as cardiomyocytes [4, 10–13]. In order to elucidate the molecular mechanisms that control $H_2O_2$-mediated effects in skeletal myoblasts, the second task of these studies was to relate the apoptotic capacities of $H_2O_2$ to the activation of molecules of MAPKs – ERK, JNK and p38 MAPK. The activation of MAPKs was investigated by the Western blot analysis. In these experiments, an $H_2O_2$ concentration inducing a 40–60% cell number decrease was used. The obtained data showed that ERK and

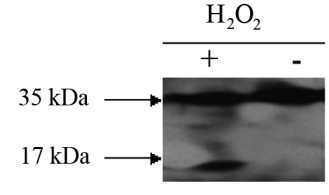


**Fig. 2.** Activation of caspase-3 after 20 h of treatment with 500 μM $H_2O_2$. Representative immunoblots from 2 experiments are shown

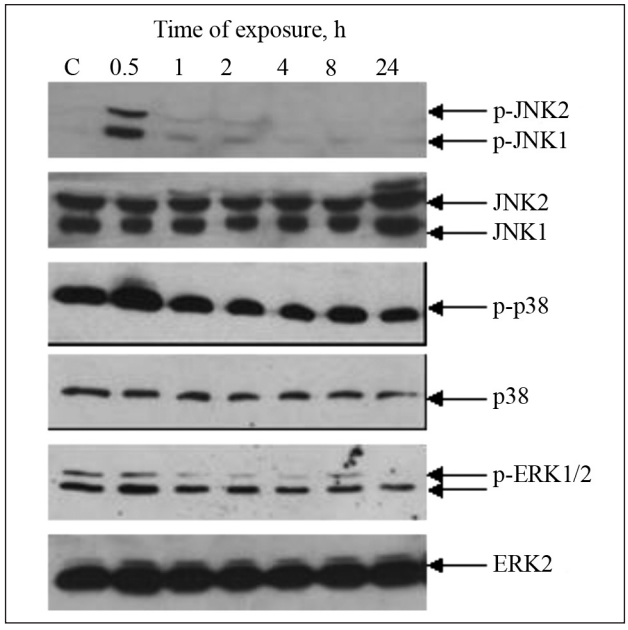


**Fig. 3.** $H_2O_2$-induced activation of JNK and p38 MAPK, but not ERK. Myogenic cells were exposed to 500 μM $H_2O_2$ for the indicated time periods. Representative immunoblots from 2 or more experiments are shown

stress-activated kinases – JNK and p38 MAPK – were activated in a different manner. $H_2O_2$ induced a significant phosphorylation of JNK and p38 MAPKs, which was transient with a peak at 30 min and returned to the basal level 1–2 h after $H_2O_2$ addition (Fig. 3) The effect of $H_2O_2$ on the phosphorylation of ERK was negligible. No variation in the total amount of MAPKs was detected. A detailed analysis of the phosphorylation of JNK and p38 MAP kinases after a brief $H_2O_2$ treatment proved their transient activation (Fig. 4).

It has been proposed that activation of JNK and p38 kinases contributes to cell death, whereas activation of ERK1/2 contributes to protection against cell injury in multiple organs [14]. To investigate the biological role of MAPKs in $H_2O_2$-induced myogenic cell apoptosis, we used the specific inhibitors of ERK, JNK and p38 MAPK – UO126, SP600125 and SB 203580, respectively.

We have found that pretreatment with UO126, SP600125 and SB203580 increased $H_2O_2$-induced apoptosis (Fig. 5). These results suggest a protective role of ERK, JNK and p38 MAP kinases in myogenic cell apoptosis induced by oxidative stress exemplified by $H_2O_2$.

Numerous reports have documented changes of kinase signaling pathways in ROS-stressed cells and tissues, including cardiac myocytes and intact myocardium [15, 16]. It has been suggested that different durations of MAPKs phosphorylation are associated with their different actions in response to oxidative stress [17–19]. Moreover, high levels of oxidative stress promote cardiac myocyte death, though lower levels are potentially cytoprotective. It has been shown that JNK and p38 MAPK are both activated by ischaemia-reperfusion in the intact heart, but their role is not definitely elucidated. Our results suggested protective actions of JNK and p38 MAPK as well as ERK under some circumstances. According to the literature data, duration of JNK and p38 activation is the critical factor in determining cell fate after various treatments: a transient activation of JNK1/2 generally correlates with a survival phenotype, whereas a prolonged activation of this kinase contributes to apoptosis. The results of the present study demonstrate that the transient activation of p38 and JNK MAP kinases might be responsible for their antiapoptotic action.

A better understanding of ROS-mediated signaling mechanisms in stem cells may enable the development of new targeted therapy strategies to improve cardiomioplasticity. Therefore, fu-

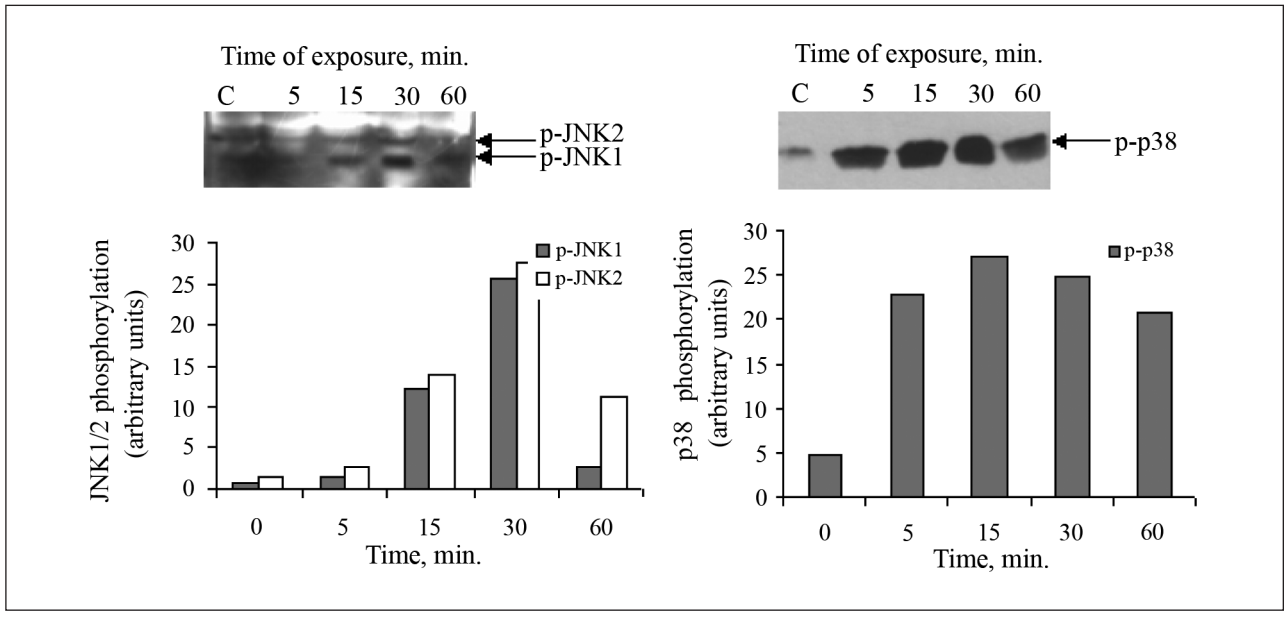


**Fig. 4.** Activation of JNK and p38 MAP kinases after a brief exposure to 1 mM $H_2O_2$. The intensity of bands on a Western blot was quantified by using Image J. Representative immunoblots from two experiments are shown

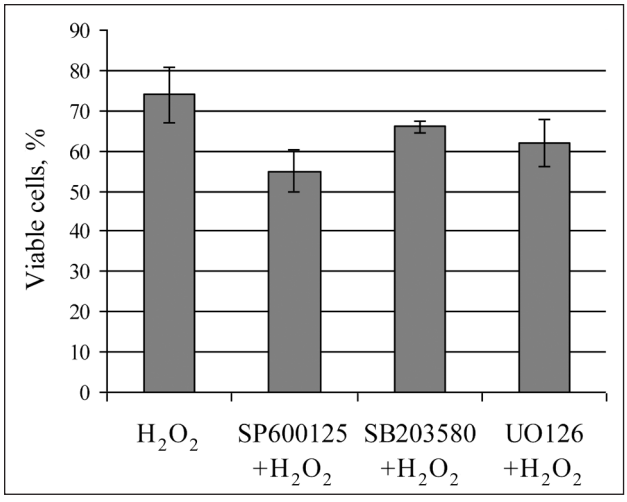


**Fig. 5.** Involvement of JNK, p38 and ERK in $H_2O_2$-induced myogenic cell apoptosis. Myogenic cells were pretreated with 10 μM U0126, 20 μM SB203580 (SB), or 20 μM SP600125 (SP) for 30 min before adding 500 μM $H_2O_2$ for 20 h. Results are expressed as the percentage of viable cells after 20 h versus viable cells before treatment. Data are expressed as mean ± SD from at least three measurements

ture studies will be directed towards identifying the downstream effectors of stress-activated kinases responsible for mediating their effects on cell survival after stress.

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## MITOGENŲ AKTYVINAMŲ BALTYMŲ KINAZIŲ VAIDMUO VANDENILIO PEROKSIDO SUKELTOJE MIOGENINIŲ LĄSTELIŲ APOPTOZĖJE

*Santrauka*

Viena svarbiausių ląstelių transplantacijos į pažeistą širdį problemų – transplantuotų ląstelių išgyvenimas bei integracija širdies audinyje. Įvykus infarktui, dėl apoptozės ir nekrozės žūva kardiomiocitai, prasideda uždegiminis procesas, kurio metu išsiskiria įvairios bioreaktyvios molekulės – citokinai, reaktyvūs deguonies dariniai (RDD), turintys įtakos kardiomiocitų bei į širdį įsodintų ląstelių išgyvenimui. Šiame darbe tyrėme mitogenų aktyvinamų baltymų kinazių (MAPK) vaidmenį vieno iš RDD atstovų – vandenilio peroksido – sukeltos miogeninių ląstelių apoptozės metu. Pradžioje nustatėme, kad mažos vandenilio peroksido koncentracijos (100 μM) stabdo tirtų ląstelių proliferaciją, bet nesukelia jų žūties; didesnės (500 μM) skatina apoptozinę žūtį. 1000 μM vandenilio peroksido koncentracija paveiktoje miogeninių ląstelių populiacijoje rasta dalis dėl nekrozės žuvusių ląstelių.

MAPK šeimos, kuriai priklauso ekstraląstelinio signalo reguliuojamos kinazės 1/2 (ERK1/2), c-Jun N-galo kinazės 1/2 (JNK 1/2) ir p38 MAP kinazė, aktyvinimas yra svarbus įvairių procesų, vykstančių širdyje, patogenezei. Mūsų duomenimis, apoptozę sukeliančios vandenilio peroksido koncentracijos skatina trumpalaikę, apie 1–2 val., JNK ir p38 MAPK aktyvaciją, tuo tarpu ERK kinazės fosforilinimo lygio padidėjimas nebuvo nustatytas. Specifinis šių kinazių slopinimas rodo galimą jų apsauginį vaidmenį vandenilio peroksido sukeltoje miogeninių ląstelių apoptozėje.