Protective effect of M_3 receptor on H_2O_2-induced apoptosis of rat myocardial cells *in vitro*

LIU Yan[1] , SUN Hong-li[1] , WU Hong[1] , GAO Yan-hui[2] , LI Hu-lun[3] , YANG Bao-feng[1]*

(1 . *Department of Pharmacology , Bio-pharmaceutical Key Laboratory of Heilongjiang Province-Incubator of State Key Laboratory , 2 . Institute of Keshan Disease , 3 . Department of Neurobiology , Harbin Medical University , Harbin 150086 , China*)

Abstract: **Aim** To observe the effect of activation of M_3 receptor on H_2O_2 induced apoptosis in cultured rat myocytes and to investigate its possible mechanisms. **Methods** Isolated neonatal cardiomyocytes were cultured. Morphologic changes were observed by microscopy. The apoptosis in cardiomyocyte was detected by terminal deoxynucleotide transferase directed d-UTP nick and end labeling (TUNEL) assay. The expression of apoptosis-related protein in Bcl-2 and Fas was measured by immunohistochemistry assay. $[Ca^{2+}]_i$ in single cardiomyocyte loaded with Fluo 3-AM was measured by confocal microscope. **Results** H_2O_2-mediated myocyte apoptosis was attenuated by M_3 receptor agonist choline (10 mmol·L^{-1}) . Pretreatment of cardiac myocytes with choline also increased Bcl-2 , decreased Fas expression , and inhibited the increase in FI value of $[Ca^{2+}]_i$ in H_2O_2-stimulated cardiac myocytes. However , blockade of M_3 receptor by 4DAMP (10 nmol·L^{-1}) completely inhibited the effects of choline on H_2O_2-stimulated cardiac myocytes. **Conclusion** Activation of M_3 receptor showed protective effect on H_2O_2-induced apoptosis in cultured rat myocytes and this effect might be related to modulating the expression of some genes including Bcl-2 and Fas as well as the downregulation of $[Ca^{2+}]_i$.

Key words: M_3 receptor; apoptosis; cultured myocyte; hydrogen peroxide; *in situ* nick-end labeling; confocal microscope; calcium

CLC number: R967 **Document code**: A **Article ID**: 0513 - 4870(2004)11 - 0887 - 05

M_3受体对体外H_2O_2诱导大鼠心肌细胞凋亡的保护作用

刘 艳[1] , 孙宏丽[1] , 吴 红[1] , 高彦辉[2] , 李呼伦[3] , 杨宝峰[1]*

(哈尔滨医科大学 1 . 药理教研室 , 黑龙江省生物医药重点实验室-省部共建国家重点实验室培育基地 , 2 . 克山病研究所 , 3 . 神经生物教研室 , 黑龙江 哈尔滨 150086)

摘要: 目的 探讨 M_3受体激动对 H_2O_2诱导的大鼠培养心肌细胞凋亡的作用 ,进一步阐明其机制。方法 末端标记法 (TUNEL) 进行细胞凋亡检测 ;免疫组化方法检测 Bcl-2 和 Fas 的表达 ;共聚焦显微镜观察$[Ca^{2+}]_i$荧光强度变化。结果 M_3受体激动剂胆碱(10 mmol·L^{-1}) 可减少 H_2O_2诱导的心肌细胞凋亡的数量 ,并可增加心肌 Bcl-2 的表达 ,减少 Fas 表达 ,抑制 H_2O_2诱导的$[Ca^{2+}]_i$荧光强度的升高。但预先应用 4DAMP (10 nmol·L^{-1}) 阻断 M_3受体可逆转胆碱作用。结论 激动 M_3受体对 H_2O_2诱导的心肌细胞凋亡有保护作用 ,其机制可能与 Bcl-2 和 Fas 表达以及下调$[Ca^{2+}]_i$有关。

关键词: M_3受体; 细胞凋亡; 培养的心肌细胞; 过氧化氢; 原位缺口末端标记; 共聚焦显微镜; 钙

Apoptosis is a type of programmed cell death that is a central feature of normal tissue development during embryonic development and normal tissue turnover. Accumulating evidence suggests that terminally differentiated adult cardiac myocytes undergo apoptosis in

Received date : 2003-12-08 .
Foundation item : National Science Fundation of China (30271599) .
Corresponding author Tel : 86 - 451 - 86671354 ,
Fax : 86 - 451 - 86669482 ,
E-mail : Yangbf@ems.hrbmu.edu.cn

various animal models , which include models of rapid ventricular pacing [1], dilatation of the heart and heart failure [2]. Thus , to explore the use of antiapoptotic agent inhibiting cardiac apoptosis is critical to the treatment of many myocardic diseases .

To date , at least five different subtypes of mAChRs have been pharmacologically defined in primary tissues , designed M_1 , M_2 , M_3 , M_4 , and M_5 . Each subtype has its own characterized distribution and function . Previous studies have provided evidence suggesting that M_3 receptor is present in the heart[3,4]. Activation of cardiac M_3 receptor also produced changes of physiological functions of the rat and rabbit hearts , such as negative inotropic and chronotropic effect[5]. Recently , it has been shown that M_3 receptor agonist choline plays an important role in the prevention of many pathologic conditions , such as cirrhosis of the liver , arteriosclerosis , certain deficiencies of brain function and memory , and Ca^{2+} induced ventricular tachycardia[6]. Furthermore , the M_3 receptor agonist choline has been demonstrated to be able to prevent occurrence of apoptosis in cultured cerebral neurons[7]. To our knowledge , whether the cardiac M_3 receptor could also inhibit apoptosis in heart has not been characterized . Therefore , the present study was conducted to determine the protective effects of activation of M_3 receptor on H_2O_2- induced apoptosis in cultured rat myocytes and to further investigate its possible mechanism .

Materials and methods

Reagents Fluo 3-acetoxymethyl (AM) ester (Molecular Probes Eugene OR , USA) was dissolved in Me_2SO (1 g · L^{-1}) and stored at 20 ℃ in the dark . Rabbit anti-rat monoclonal antibodies (including Bcl-2 and Fas) were prepared by Beijing Zhongshan Biological Technology Co . Immunohistochemistry kit and TUNEL kit were provided by Wuhan Boster Biological Technology Co . Choline and 4-diphenylacetoxy-*N*-methylpiperidine-methiodide (4DAMP) were donated by the Research Center , Montreal Heart Institute , Canada .

Cell culture and drug treatment The hearts were taken from 2 - 3 d Wistar rats provided by the Experimental Animal Center of Harbin Medical University (Grade II , Certification No . 0921) . Then cut into pieces of about 1 mm^3 which was digested into cell suspension by 0.2 % collagenase . After centrifugation , cells were resuspended in RPMI-1640 containing 20 % fetal calf serum (FCS) . Cardiomyocytes accounted for about 90 % after repeated purification . The number of cells in suspension was adjusted to 5×10^8 · L^{-1} and cells were plated on 25 mm round coverslip on the bottom of 6-well multidish for 48 h in CO_2 incubator and the cells were treated with saline , H_2O_2 (50 μmol · L^{-1}) alone , H_2O_2 plus choline (10 mmol · L^{-1}) , or 4DAMP (10 nmol · L^{-1}) in addition to H_2O_2 and choline in serum-free medium for 24 h . All preparations were ready for apoptosis investigation , protein expression , and measurement of $[Ca^{2+}]_i$.

Determination of apoptosis Cardiomyocyte apoptosis was investigated with TUNEL method for the percentage of apoptosis cells . After that , the cardiomyocytes were stained with hematoxylin for the observation with light microscope .

Detection of Bcl-2 and Fas protein expression Bcl-2 and Fas protein expression was surveyed by immunohistochemistry and computer image analysis system . Brown staining in cell was evaluated as positive expression , which was examined by calculating the ration of mean optic density and area in positive stain , namely , positive expressive index (PEI) .

Measurement of $[Ca^{2+}]_i$[8] Cultured single cardiomyocyte lined on the coverslip were washed twice with calcium-free phosphate buffer solution , and loaded in Fluo 3-AM (10 μmol · L^{-1}) working solution containing 0.03 % F-127 at 37 ℃ for 40 min , and washing with Tyrode solution (1.8 mmol · L^{-1}) to remove extracellular Fluo 3-AM . Then , the preparation was mounted in the cell chamber with Tyrode solution 300 μL . The fluorescent intensity was detected by confocal microscope (Insight Plus-IQ , Meridian , MI , USA) with 40 objective and 488 nm argon ion laser for excitation and 530 nm for emission at room temperature . The fluorescent images of $[Ca^{2+}]_i$ were collected from four groups . The $[Ca^{2+}]_i$ concentration change was represented by fluorescent intensity .

Data analysis All results are expressed as $\bar{x} \pm s$. Unpaired data were evaluated by Student *t*-test . A value of $P < 0.05$ was considered significant .

Results

1 Effect of M_3 receptor on morphologic changes

With reversed light microscope , the normal control group of cells were lined on the coverslip with spindle or elliptic shape and the cells stimulated with choline showed no obvious morphologic change . After incubated with H_2O_2 (50 μmol · L^{-1}) for 24 h , some cardiomyocytes

changed into round shape with refractivity and lost the connection with surrounding cells. Pretreatment of cells with choline (10 mmol·L^{-1}) attenuated the injury of H_2O_2 in cardiomyocytes. 4DAMP (10 nmol·L^{-1}) reversed the effect of choline on H_2O_2-stimulated cardiac myocytes.

2 Effect of M_3 receptor on apoptosis in cardiomyocytes

After hematoxylin stain, the color of normal cardiomyocytes showed a light blue (TUNEL negative) and apoptosis cells (TUNEL positive) were light brown under the light microscope. A representative quantitative data was shown in Table 1. Under our experimental conditions, nearly no apoptosis cells were found in the control group. Stimulation with H_2O_2 (50 μmol·L^{-1}) for 24 h markedly increased the number of TUNEL-positive cells. Fewer myocardial cells treated with choline (10 mmol·L^{-1}) plus H_2O_2 were positive by TUNEL staining compared with the cells treated with H_2O_2 alone. 4DAMP (10 nmol·L^{-1}) in addition to H_2O_2 and choline increased the number of TUNEL- positive myocytes compared with the cells treated with choline plus H_2O_2.

Table 1 Effect of M_3 receptor on the apoptosis rate of cardiomyocytes

Group	Apoptosis rate / %
Control	0.9 ± 0.5
H_2O_2	13.8 ± 1.3**
Choline + H_2O_2	3.4 ± 0.5##
4DAMP + choline + H_2O_2	11 ± 8$$

$n = 8$, $\bar{x} \pm s$. *$P < 0.01$ *vs* control; #$P < 0.01$ *vs* H_2O_2; $$$P < 0.01$ *vs* choline + H_2O_2. Apoptosis in cardiac myocytes by TUNEL method. Neonatal rat cardiac myocytes were treated with saline as a control, with choline (10 mmol·L^{-1}) in the presence or the absence of H_2O_2 (50 μmol·L^{-1}), or with 4DAMP (10 nmol·L^{-1}) in the presence of choline and H_2O_2 for 24 h. Compared with the H_2O_2 group, M_3 receptor agonist choline reduced the number of TUNEL-positive cells (apoptosis cells) in cardiomyocytes of rats stimulation with H_2O_2 for 24 h. However, M_3 receptor antagonist 4DAMP reversed the effect of choline

3 Effects of the activation of M_3 receptor on expression of apoptosis-related factors

The control of programmed cell death is dependent on a balance between inhibitors and inducers of apoptosis. To further confirm the mechanism by which M_3 receptor exerts antiapoptotic effects on oxidant stress-induced apoptosis in cardiac myocytes, we detected the expression of Bcl-2 and Fas by immunohistochemistry.

Choline (10 mmol·L^{-1}) stimulation induced the expression of Bcl-2 protein in cardiac myocytes by (4.1 ± 1.6) fold compared with cells treated with H_2O_2 alone. Expression of Bcl-2 protein were reduced by (2.4 ± 0.2) fold in group 4DAMP (10 nmol·L^{-1}) compared with that of the choline group.

H_2O_2 induced the expression of Fas. Pretreatment with choline inhibited the induction of expression of Fas by H_2O_2. However, blockage of M_3 receptor by 4DAMP reversed the inhibition of expression of Fas by choline in these cells (Table 2).

Table 2 Effects of the activation of M_3 receptor on Bcl-2 and Fas protein expression in H_2O_2-stimulated cardiomyocytes

Group	PEI / %	
	Bcl-2	Fas
Control	2.7 ± 0.9	1.9 ± 0.9
H_2O_2	1.1 ± 0.8**	6.1 ± 1.3**
Choline + H_2O_2	4.5 ± 1.3##	2.2 ± 0.8##
4DAMP + choline + H_2O_2	1.9 ± 0.6$$	5.6 ± 1.7$$

$n = 8$, $\bar{x} \pm s$. *$P < 0.01$ *vs* control; #$P < 0.01$ *vs* H_2O_2; $$$P < 0.01$ *vs* choline + H_2O_2. Bcl-2 and Fas protein expression in cardiac myocytes by immunohistochemistry. Neonatal rat cardiac myocytes were treated with saline as a control, with choline (10 mmol·L^{-1}) in the presence or the absence of H_2O_2 (50 μmol·L^{-1}), or with 4DAMP (10 nmol·L^{-1}) in the presence of choline and H_2O_2 for 24 h. Compared with the H_2O_2 group, M_3 receptor agonist choline increased myocardial expression of Bcl-2 protein and reduced Fas protein in cardiomyocytes of rats stimulation with H_2O_2 for 24 h. However, M_3 receptor antagonist 4DAMP reversed the effect of choline. PEI: Positive expressive index

4 Effect of M_3 receptor on $[Ca^{2+}]_i$ mobilization

Because increasing evidence recently indicated that Ca^{2+} played important role in the execution of apoptosis in a number of different cell types. We then determined the possible role of Ca^{2+} in the antiapoptotic effects of M_3 receptor on cardiomyocytes stimulated with H_2O_2. After incubation with H_2O_2 (50 μmol·L^{-1}) for 24 h, the fluorescent intensity (FI) of $[Ca^{2+}]_i$ in cardiac myocytes increased markedly ($P < 0.01$). The FI value of $[Ca^{2+}]_i$ in cells treated with choline (10 mmol·L^{-1}) plus H_2O_2 was maintained at a lower level than that in cells treated with H_2O_2 alone ($P < 0.01$). 4DAMP (10 nmol·L^{-1}), in addition to H_2O_2 and choline, increased the FI value of $[Ca^{2+}]_i$ compared with the cells treated with choline plus H_2O_2 ($P < 0.01$, Table 3).

Table 3 Effect of M_3 receptor on $[Ca^{2+}]_i$ mobilization in cultured cardiomyocytes

Group	$[Ca^{2+}]_i$/FM	*n*
Control	324 ± 76	12
H_2O_2	592 ± 110**	11
Choline + H_2O_2	366 ± 76##	21
4-DAMP + choline + H_2O_2	465 ± 102$$	12

$\bar{x} \pm s$. *$P < 0.01$ *vs* control; #$P < 0.01$ *vs* H_2O_2; $$P < 0.01$ *vs* choline + H_2O_2. $[Ca^{2+}]_i$ mobilization in cardiac myocytes. Neonatal rat cardiac myocytes were treated with saline as a control, with choline (10 mmol·L^{-1}) in the presence or the absence of H_2O_2 (50 μmol·L^{-1}), or with 4-DAMP (10 nmol·L^{-1}) in the presence of choline and H_2O_2 for 24 h. Compared with the H_2O_2 group, M_3 receptor agonist choline decreased FI value of $[Ca^{2+}]_i$ in cardiomyocytes of rats stimulation with H_2O_2 for 24 h. However, M_3 receptor antagonist 4-DAMP reversed the effect of choline

Discussion

Increasing evidence shows that H_2O_2 plays an important role in the pathogenesis of a variety of cardiac diseases. The present work reveals that in cultured cardiomyocytes, H_2O_2 induced significant apoptosis without cytolytic change determined by TUNEL method, suggesting that DNA fragmentation occurred in apoptosis cardiomyocytes. This is consistent with other studies which suggest that oxidative stress induce programmed cell death and H_2O_2 and O^{2-} have been shown to induce apoptosis in cultured rat neonatal myocytes[9]. Using primary neonatal rat cardiac myocytes, we also showed that the activation of M_3 receptor blocked H_2O_2-induced increase in the TUNEL-positive cells. However, 4-DAMP, a selective blocker of M_3 receptor, completely inhibited the protective effects of choline on H_2O_2-induced apoptosis, strongly suggesting that the activation of M_3 receptor has an antiapoptotic effect on oxidant stress-induced apoptosis in cardiac myocytes.

The induction of apoptosis is associated with the expression and/or activation of specific proteins, resulting in the execution of the apoptotic program within the affected cells. In general, a plethora of different signaling pathways could be involved in apoptosis, depending on the stimulus and/or type of cells affected. To specify the signaling pathway in M_3 receptor antiapoptosis in cardiomyocytes, we first determined the protein levels of well-known apoptosis-related factors, such as Bcl-2 and Fas. Bcl-2 gene product is a 25 kD membrane protein that functions to prevent apoptosis by various stimuli, for example it can block or restrict apoptosis induced by Fas[10]. Prevention of apoptosis by increased Bcl-2 expression has also been shown in adult cardiac myocytes. Fas, a 37 kD protein, is associated with the cardiomyocyte sarcolemma and probably represents the first step in the initiation of apoptosis. Fas belongs to the tumor necrosis factor receptor super family and are known to play a critical role in the induction of apoptosis. The present study demonstrated that stimulation of cardiac M_3 receptor with choline increased the expression of Bcl-2 protein and decreased Fas in H_2O_2-stimulated cardiac myocytes. The upregulated Bcl-2 expression and downregulated Fas expression may not be the only mechanism for the antiapoptotic effects of choline. However, the potent ability of Bcl-2 to block apoptosis in many cell types suggests that this modulation is, at least in part, involved in the antiapoptotic effects of M_3 receptor.

Ca^{2+}, an important second messenger, was involved in the regulation and metabolism in cytoplasm, gene transcription, cell differentiation and reproduction, and also apoptosis. The results with confocal microscope showed that $[Ca^{2+}]_i$ was mobilized in myocytes treated with H_2O_2, suggesting that $[Ca^{2+}]_i$ was involved in the process of H_2O_2-induced apoptosis in cultured cardiomyocyte. The increased Ca^{2+} in cytoplasm can elevate Ca^{2+} in the nucleus leading to activation and expression of some promoter genes involved in apoptosis, and the inhibition of the expression of survival genes, such as Bcl-2 gene[11], thus the activation of irreversible death process, for instance, the activation of endogenous nucleic endonuclease and glutaminase which can result in cell phenotypic change and cell death. Previous studies demonstrated that choline activates Ik_{M3} which could indirectly decrease calcium entry because of accelerated repolarization and directly reduce Ca^{2+} entry[6]. The present study showed that choline inhibited the H_2O_2-induced increase in FI value of $[Ca^{2+}]_i$ and 4-DAMP completely inhibited this effect of choline in H_2O_2-stimulated cardiac myocytes, suggesting that one mechanism of the antiapoptotic effect of activation of M_3

receptor is due to the downregulation of $[Ca^{2+}]_i$.

Taken together, our results demonstrated that the activation of M_3 receptor produced protective effects on H_2O_2-induced apoptosis in cultured rat cardiomyocyte. Bcl-2, Fas and Ca^{2+} were involved in and played important role in the process.

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