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1-Methyl-3-isobutylxanthine delays apoptosis induced by deprivation of growth factors in vascular endothelial cells

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KEY WORDS 1-methyl-3-isobutylxanthine; apoptosis; umbilical veins; vascular endothelium; cultured cells; cell survival; DNA fragmentation; acidic fibroblast growth factor.

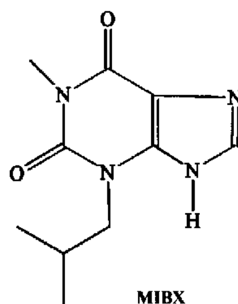
ABSTRACT

AIM: To investigate the effects of 1-methyl-3-isobutylxanthine (MIBX) on apoptosis induced by deprivation of acidic fibroblast growth factor (aFGF) and serum in vascular endothelial cells (VEC). **METHODS**: Nuclear fragmentation was observed by fluorescence microscopy. Viability was determined by counting the cells that attached to dishes after treatments. DNA fragmentation was measured by agarose gel electrophoresis. **RESULTS**: The cells deprived of aFGF and serum were treated with MIBX 25 – 200 μmol/L for 3, 6, 9, and 12 h, respectively. Morphological changes including the formation of apoptotic bodies and DNA fragmentation of these cells were significantly suppressed by IBMX 50-200 μmol/L at 3 h and 6 h. But after 12-h treatment, no difference was observed between the treated and untreated cells. **CONCLUSION**: MIBX delays apoptosis of vascular endothelial cells induced by deprivation of aFGF and serum.

INTRODUCTION

As we know, vascular degeneration is as important as angiogenesis in growth, carcinogenesis, homeostasis, and some kinds of disease[1]. Vascular endothelial cells (VEC) play important roles in the formation of blood vessels and their degeneration, and apoptosis of these cells have a positive role in the control of vascular degeneration which results in many kinds of diseases[2]. Therefore, it is useful to look for new drugs that inhibit VEC apoptosis.

1-Methyl-3-isobutylxanthin (MIBX) is a potent inhibitor of cyclic nucleotide phosphodiesterase, and it has been used in studies of tumor colony formation[3]. To gain more information on the possible therapeutic potential of MIBX, this paper was to study whether MIBX could affect VEC apoptosis.



MATERIALS AND METHODS

Reagents MCDB-105 medium was purchased from Kyokuto Pharmaceutical Industries, Tokyo, Japan. Fetal bovine serum (FBS) was purchased from Wako Industries, Tokyo. Fibroblast growth factor (FGF) was extracted from bovine brains in our laboratory by the method of Lobb and Fett[4]. MIBX was purchased from J & K China Chemical Ltd, Beijing, China. All other reagents were of AR grade.

Cell cultures Human umbilical vein endothelial cells (HUVEC) were obtained by the method of Jaffe[5]. The cells were cultured on gelatin-coated plastic dishes in MCDB-105, supplemented with 10 % FBS and aFGF 70 μg/L (as well as heparin 100 mg/L) at 37 ℃ in 5 % CO_2 + 95 % air. Cells with a population doubling level of 15 to 25 were used.

Viability assay When cultured cells reached confluence the cells were washed once with MCDB-105

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medium and replaced with the aFGF- and serum-free medium. The cells were incubated with or without MIBX. Trypsinized cells were counted on a Coulter counter after 3, 6, 9, 12, and 15 h. Detached cells were washed away before the treatment with trypsin. The cells that remained attached to dishes after washing away of blebs were not stained by erythrosin B (5 g/L, Sigma) and were therefore, regarded as living cells.

Nuclear fragmentation assay Cells after treatment were washed once with PBS (phosphate-buffered saline), fixed with 1 % glutaraldehyde solution at 25 ℃ overnight, centrifuged, and resuspended in PBS, and then stained with Hoechst 33258 at the concentration of 1 mol/L for 20 min. After three washes with PBS, the cells were mounted onto slides for analysis under a fluorescence microscope.

Analysis of DNA fragmentation Cells after treatment were harvested, and incubated in a digestion buffer that contained proteinase K 0.2 g/L at 50 ℃ overnight. The cellular DNA was extracted once with phenol and once with a mixture of phenol, chloroform, and 3-methyl-1-butanol (25:24:1, v:v:v). After digestion by RNase (final concentration 0.6 g/L) at 37 ℃ for 30 min, the samples were subjected to electrophoresis on a 2 % agarose gel in Tris-acetate buffer. The gel was then stained with ethidium bromide and photographed on a UV transilluminator.

Statistics Data were expressed as $\bar{x} \pm s$ and analyzed by t test.

RESULTS AND DISCUSSION

Apoptosis of VEC is induced by deprivation of growth factors[6,8]. Here, we have used this apoptosis inducing system to study the effect of MIBX on VEC apoptosis. After deprivation of aFGF and serum, the cells gradually started to round up and eventually became detached from the dish and floated in the medium, then apoptotic bodies were formed from these cells[6]. When VEC were exposed to MIBX 25 – 200 μmol/L in the absence of aFGF and serum for 6 h, the numbers of cells (90 %-99 %) attached to the dish were higher than those of untreated cultures (88%). The effect of MIBX was dose-dependent ($P<0.05$) (Tab 1).

The inhibitory effect of MIBX on the process of VEC apoptosis was observed within 9 h at 200 μmol/L, henceforth there was no significant difference in viability between MIBX-treated and untreated cells (Tab 2).

These results showed that MIBX inhibited the apoptotic process of VEC at an early stage.

Tab 1. Effect of MIBX on VEC apoptosis. The numbers of cells remaining on the dishes were counted 6 h after deprivation of aFGF and serum and treatment with MIBX. $n=8$ experiments. $\bar{x} \pm s$. $^aP>0.05$, $^bP<0.05$, $^cP<0.01$ vs control.

MIBX/μmol·L^{-1}	Viability/%
0	88.2 + 1.5
25	89.8 ± 2.0^a
50	93.3 ± 1.7^b
100	94.7 ± 1.2^b
200	99.4 ± 1.0^c

Tab 2. Time course of MIBX effect on VEC apoptosis at 200 μmol/L. The cells remaining on the dishes were counted 3, 6, 9, 12, and 15 h after the start of treatment. $n=8$ experiments. $\bar{x} \pm s$. $^aP>0.05$, $^bP<0.05$, $^cP<0.01$ vs control.

Time/h	Viability/%	
	Control/%	MIBX-treated/%
3	95.1 ± 2.0	98.6 ± 1.0^b
6	88.3 ± 1.5	97.8 ± 1.4^c
9	84.7 ± 2.1	92.4 ± 1.6^c
12	81.9 ± 1.2	87.3 ± 2.1^a
15	80.2 ± 1.6	83.1 ± 1.8^a

The effect of MIBX on morphological changes, (apoptotic body formation) was examined with fluorescence microscope and it was observed that nuclear fragmentation was inhibited by MIBX 200 μmol/L at 6 h (Fig 1). Agarose gel electrophoresis of DNA from the cells treated with MIBX 50-200 μmol/L revealed that DNA fragmentation induced by deprivation of aFGF and serum was supressed by MIBX (Fig 2).

Cyclic nucleotide phosphodiesterase is an important enzyme by which cAMP is cleaved, resulting in a decline in intracellular cAMP level. MIBX is used as a potent inhibitor of this enzyme to elevate the levels of intracellular cAMP[3]. cAMP might mediate some of the signals that regulate apoptosis in thymocytes[8]. As we know, in different types of cells, there are various pathways of apoptosis signal transductions, for example, Fas ligation triggers apoptosis in macrophages but not in endothelial cells[9]. So it is very interesting to understand the specific apoptosis signal transductions in a given type of cells. So far there is no report about the role of cAMP in regulation of VEC apoptosis and our data indicate that

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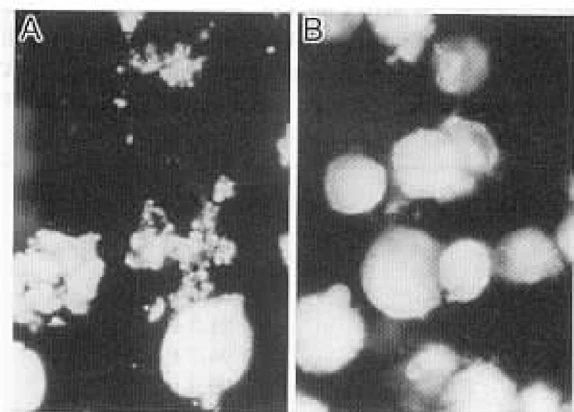

Fig 1. Fluorescence of VEC stained with Hoechst 33258. A: Cells deprived of aFGF and serum for 6 h. B: Cells deprived of aFGF and serum, and treated with MIBX 200 μmol/L for 6 h (×400).

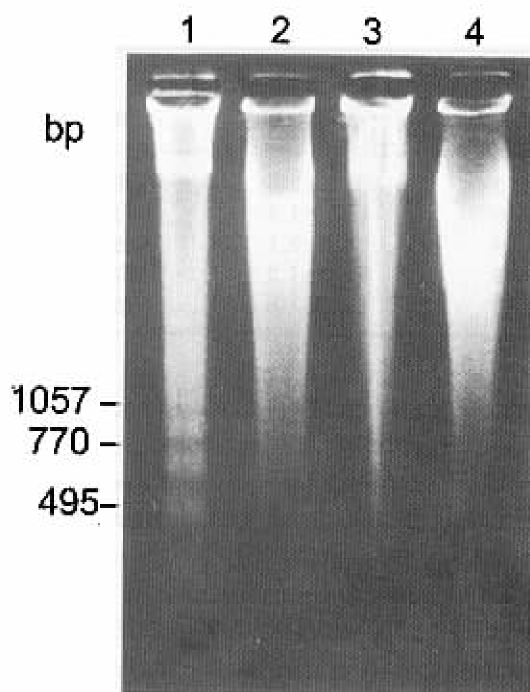

Fig 2. DNA fragmentation of VEC treated with MIBX for 6 h. (1) DNA from cells deprived of aFGF and serum; (2)-(4) DNA from cells deprived of aFGF and serum, and treated with MIBX 50, 100, and 200 μmol/L, respectively.

cAMP might be involved in apoptosis signaling in VEC.

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1-甲基-3-异丁基黄嘌呤延迟去除生长因子诱导的血管内皮细胞凋亡

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关键词 1-甲基-3-异丁基黄嘌呤; 细胞凋亡; 脐静脉; 血管内皮; 培养的细胞; 细胞存活; DNA 断片; 酸性成纤维细胞生长因子

目的: 研究 1-甲基-3-异丁基黄嘌呤(MIBX)对去除生长因子(aFGF 和血清)诱导的血管内皮细胞凋亡的影响. **方法:** 通过细胞存活率的分析,荧光显微技术和 DNA 凝胶电泳等方法,检测 MIBX 对细胞凋亡的影响. **结果:** 用 25 – 200 μmol/L 的 MIBX 处理培养在无 aFGF 和血清的培养液中的血管内皮细胞, 50 – 200 μmol/L 的 MIBX 在处理 6 h 明显抑制了凋亡小体的形成和 DNA 的片断化. 但是同样浓度的 MIBX 处理细胞 12 h 以后, 处理组和对照组之间无明显差别. **结论:** MIBX 延迟去除 aFGF 和血清诱导的血管内皮细胞凋亡.

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