Effects of MN-9202 on platelet aggregation, 5-HT release, TXB_2 synthesis, and calcium mobilization in rabbit platelets *in vitro*[1]

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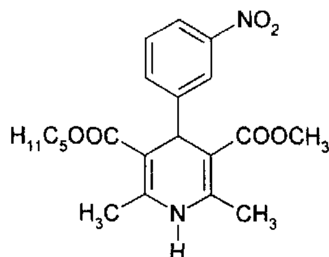
KEY WORDS calcium channel blockers; MN-9202; platelet aggregation; calcium; serotonin; thromoxane B_2; Fura-2

ABSTRACT

AIM: To study the effects of MN-9202, a new effective Ca^{2+} channel blocker, on platelet aggregation, 5-HT and TXB_2 release, and calcium transport induced by platelet activators. **METHODS**: The mobilization of cytosolic-free calcium induced by thrombin in washed platelets was observed by Ca^{2+}-sensitive fluorescent indicator, Fura-2 AM and time scan measurement. Aggregation induced by ADP and thrombin in rabbits citrate platelet-rich plasma (PRP) was measured by aggregometer. 5-HT and TXB_2 were assayed by HPLC/ECD and RIA, respectively. **RESULTS**: MN-9202 inhibited platelet aggregation induced by ADP and thrombin in a concentration-dependent manner. MN-9202 1 $\mu mol \cdot L^{-1}$ inhibited release of 5-HT in PRP induced by collagen at 15 $mg \cdot L^{-1}$ (113 ± 15 *vs* 178 ± 18, $P < 0.05$), however, MN-9202 did not have effect on 5-HT secreted by high dose of collagen. MN-9202 0.1 and 1 $\mu mol \cdot L^{-1}$ blocked extracellular calcium influx and sarcoplasmic calcium release, and the suppression on extracellular calcium influx was more obvious. Furthermore, treatment with MN-9202 0.01, 0.1, and 1 $\mu mol \cdot L^{-1}$ markedly decreased ADP-induced TXB_2 (pg/10^8 platelet) release from PRP (906 ± 200, 881 ± 131, and 793 ± 169 *vs* 1264 ± 202, $P < 0.01$). **CONCLUSION**: MN-9202 acts as an effective Ca^{2+} antagonist and blocks platelet activation by inhibiting platelet Ca^{2+} influx and arachidonic acid metabolism.

INTRODUCTION

It has been demonstrated that platelets play an important role in many cardiovascular diseases, including vasospasm, atherosclerosis, coronary thrombosis, acute myocardial infarction, and various other ischemic diseases. Although dihydropyridines (DHP) are used widely in such therapeutical states and some compounds of DHP have been reported to have the power to inhibit platelet activation[1], the exact mechanism of DHP on platelets[2] is still controversial. Methyl pentyl 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylate (MN-9202) is a novel synthesized DHP, which has been verified for having protective effects on intestinal-ischemia reperfusion, cerebral ischemia, and thrombosis in experimental animal models[3-6]. So, using MN-9202 as a typical DHP, we investigated the effects of MN-9202 on key parameters such as aggregation, release of 5-HT, calcium mobilization, and TXB_2 synthesis in rabbits platelets *in vitro*.



Methyl pentyl 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylate (MN-9202)

MATERIALS AND METHODS

Rabbits New Zealand rabbits ($n = 30$, ♂, weighing 1.8 - 2.2 kg) were supplied by the Experimen-

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tal Animal Center, Fourth Military Medical University (Grade Ⅱ, Certificate № 08-015).

Drug and reagents MN-9202 ($C_{21}H_{26}N_2O_6$) was synthesized in our laboratory. The chemical purity was more than 98 % as determined by HPLC. Its chemical structure was verified by nuclear magnetic resonance spectrometry (NMR), mass spectrum (MS), and element analysis. It was dissolved in vehicle mixed with Me_2SO, ethanol, polyethylene glycol-400 (PEG), and distilled water (v:v:v:v = 2:3:6:9). Fura-2 AM was from Institute of Materia Medica, Chinese Academy of Medical Sciences. ADP, 5-HT, thrombin, and collagen (type Ⅰ) were purchased from Sigma, USA.

Platelet preparation and aggregation Blood was drawn from arteria carotid of narcotized rabbits into plastic syringes with 1 volume of anticoagulant ACD (citric acid 15 $g \cdot L^{-1}$, trisodium citrate 25 $g \cdot L^{-1}$, and *D*-glucose 20 $g \cdot L^{-1}$) for 6 volumes of blood. The platelet-rich plasma (PRP) was obtained by centrifugation (250 *g* for 15 min). Aggregation experiment was performed with 200 μL PRP (the concentration of PRP was adjusted to 1×10^7 platelets/L) in TYXN-91 aguloaggreggameter (Institute of Shanghai Current Machine-Electricity Technology Research). After being incubated with vehicle or MN-9202 at 37 ℃ for 3 min and stirred for 30 s, PRP was then stimulated with agonists. The final ADP and thrombin concentrations, chosen to induce 40 % of maximal aggregation [100 % = platelet-poor plasma (PPP)], were 2 $mmol \cdot L^{-1}$ and 125 $U \cdot L^{-1}$. The aggregation curve was recorded for 5 min. For each rabbit three concentrations of MN-9202 and the vehicle control were tested in PRP samples, each concentration of MN-9202 was tested in 6 to 8 animals. The effect of drug was expressed as aggregation inhibition rate (AIR %).

$$AIR\ (\%) = \frac{AIR_{Control} - AIR_{Drug}}{AIR_{Drug}}$$

Calcium mobilization studies[7] Fresh PRP was supplemented with aspirin (0.1 $mmol \cdot L^{-1}$) and then incubated with Fura-2 AM 5 $mmol \cdot L^{-1}$ at 37 ℃ for 45 min. The Fura-2 loaded platelets were collected by centrifugation at $1000 \times g$ for 20 min, washed twice and then resuspended in Ca^{2+}-free HEPES-buffered saline (HBS) containing egtazic acid 0.1 $mmol \cdot L^{-1}$ at the density of 10^{11} platelets/L and incubated with vehicle and various concentrations of MN-9202 for 6 min. Thrombin 0.2 U was added at 20 s and extracellular Ca^{2+} at 5 $mmol \cdot L^{-1}$ was supplemented at 180 s. The fluorescence of Fura-2-loaded cells was measured with Time Scan Measurement in RF-5000 spectrofluorophotometer (SHIMADZU, Japan), with excitation wavelength at 340 nm and 380 nm, emission wavelength at 500 nm, and Exit and Emi bandwidth at 5 nm and 10 nm, respectively. The scan time was 6 min. The change of $[Ca^{2+}]_i$ was expressed as F_{340}/F_{380} and calculated from the ratio of fluorescence intensities as described before[8].

Platelet release reaction of 5-HT PRP was mixed with edetic acid 2 $mmol \cdot L^{-1}$ and the platelet pellet was suspended in Ca^{2+}-Tyrode's buffer. After incubation at 37 ℃ for 20 min, the washed platelet was resuspended in Tyrode's buffer containing Ca^{2+} 1 $mmol \cdot L^{-1}$ and the cell concentrations were adjusted to about 1×10^{11} platelets/L. Then vehicle or various doses of MN-9202 were added to platelets for 3 min prior to collagen stimulation. The reaction was terminated 3 min later by mixing the sample with 100 μL edetic acid 0.05 $mmol \cdot L^{-1}$ in ice, then the platelets were centrifuged at $10\ 000 \times g$ for 5 min. The supernatant was passed through a 0.22-μm membrane filter. The filtrate 10 μL was directly injected into LC-6A reversed-phase HPLC/ECD system (SHIMADZU, Japan).

Platelet TXB_2 assay PRP was incubated with vehicle or various concentrations of MN-9202 at 37 ℃ for 3 min and stirred for 30 s. Aggregation induced by ADP was performed for 5 min then terminated by indomethacin 10 $\mu mol \cdot L^{-1}$. The tube was centrifuged at $12000 \times g$ for 5 min at 4 ℃, the supernatant was preserved at −20 ℃, then assayed with TXB_2 RIA kit (Institute of Shuzhou Thrombus Research, China).

Statistical analysis Data were expressed as $\bar{x} \pm s$, *P* was evaluated by *t* test. The effect of drug on platelet aggregation was analyzed by D/R linear regression[9].

RESULTS

Effect of MN-9202 on platelet aggregation

MN-9202 inhibited platelet aggregation induced by ADP and thrombin in a concentration-dependent manner (Tab 1). The correlation coefficient were 0.964 and 0.946, respectively. The effectiveness of MN-9202 on aggregation induced by ADP and thrombin was almost the same.

Effect of MN-9202 on $[Ca^{2+}]_i$ mobilization

When Fura-2-loaded platelets suspended in Ca^{2+}-free buffer were stimulated with thrombin, cytosolic calcium increased slightly and only a small peak was seen in the

Tab 1. Effect of MN-9202 on platelet aggregation. $n = 6 - 8$ rabbits. $\bar{x} \pm s$. $r_{ADP} = 0.964$, $r_{Thrombin} = 0.949$.

MN-9202/ $\mu mol \cdot L^{-1}$	n	Inhibitory rate/%	
		ADP	Thrombin
Vehicle	6	0	0
0.01	8	10±4	10±3
0.1	8	16±6	18±7
1	8	27±7	29±6

time scan tracings. But when extracellular Ca^{2+} was supplemented at 5 mmol · L^{-1}, cytosolic calcium increased greatly and a huge wave was recorded. Treatment with various concentrations of MN-9202 made it drop in a concentration-dependent manner. (Fig 1)

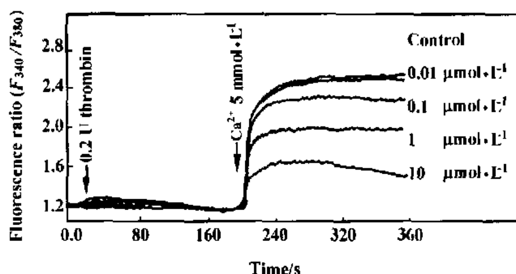

Fig 1. Concentration dependent effects of MN-9202 on Ca^{2+} release and influx of washed rabbit platelets. Trace shows the representative of at least 6 independent experiments.

Effect of MN-9202 on release of TXB_2 and 5-HT The formation of TXB_2 and the release of endogenous 5-HT was enhanced markedly by ADP and collagen, respectively. MN-9202 0.1 and 1 mmol·L^{-1} suppressed the secretion of 5-HT induced by collagen 15 mg·L^{-1}($P < 0.05$), but had no effect on that induced by a higher dose of collagen (45 mg·L^{-1}). MN-9202 at every concentration tested inhibited the release of TXB_2(Tab 2).

DISCUSSION

Previous studies indicated that the increase in cytosolic-free calcium played an important role as a second messenger in platelet activation induced by a stimulus[10]. Some of DHP could inhibit the increase in the cytosolic free Ca^{2+} concentration and cause a concentration-dependent inhibition of platelet aggregation[11]. The above observation are consistent with the results of the present study that MN-9202 inhibits both platelet activation and

Tab 2. Effect of MN-9202 on release of TXB_2 and 5-HT. $n = 6 - 10$ rabbits. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ vs vehicle control.

MN-9202/ $\mu mol \cdot L^{-1}$	TXB_2 (pg/10^8 platelet)	5-HT (mg/10^{11} platelet)	
		collagen/ 15 mg·L^{-1}	collagen/ 45 mg·L^{-1}
Vehicle	1264±202	178±18	523±41
0.01	906±200^c	154±15	530±54
0.1	881±131^c	131±25^b	501±67
1	794±269^c	129±23^b	493±49

calcium influx.

Platelet activation has three phases namely aggregation, reformation, and release reaction. Our results showed that MN-9202 inhibited ADP- and thrombin-induced platelets aggregation in a concentration-dependent manner. 5-HT, a key substance released from dense granules when platelets are activated, participates in a positive feedback regulation of platelet activation[12]. MN-9202 1 μmol·L^{-1} inhibited secretion of 5-HT in PRP induced by collagen at 15 mg·L^{-1}, however, MN-9202 had no effect on 5-HT secretion by high dosage of collagen. This indicated that the effect of MN-9202 on an over-activated platelet was limited.

Platelet aggregation is believed to be closely related to an increase in cytosolic calcium[10]. In this experiment, we observed that thrombin-induced increase in cytosolic calcium consisted of transmembrane influx of extracellular Ca^{2+} and release of plasma membrane-bound calcium, which was consistent with previous reports[11]. We used Ca^{2+}-sensitive fluorescent indicator, Fura-2 AM and Time Scan Measurement to observe the changes in cytosolic free calcium in activated-platelet of rabbits. MN-9202 blocked increase in cytosolic calcium by inhibiting influx of extracellular calcium and calcium release from plasmia reticulum. The suppression of MN-9202 on influx of extracellular Ca^{2+} is more marked than that on intracellular calcium release. It seems unlikely that inhibition of intracellular calcium release plays a major role in the anti-aggregatory activity of MN-9202.

Thromboxane, another agent released from activated platelets, follows a positive feedback regulation and is synthesized by platelet cyclo-oxygenase. It is believed that TXB_2 formation is related to the increase in the intracellular calcium and the state of platelet activation[10]. In this study, MN-9202 at concentrations tested showed marked inhibitory effects on formation of TXB_2.

In conclusion, the inhibitory effect of MN-9202 on

platelet aggregation can be attributed to its suppression on intracellur calcium, arachidonic acid metabolism, and the degranulation of platelets.

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MN-9202 对兔血小板聚集、5-HT 释放、TXB_2 合成及 Ca^{2+} 转运的影响[1]

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关键词 钙通道阻滞剂; MN-9202; 血小板聚集; 钙; 血清素; 血栓素 B_2; Fura-2

目的: 研究新二氢吡啶类钙拮抗剂 MN-9202 对兔血小板激活的影响, 并探讨其作用机制. **方法**: 以 Fura-2 AM 为荧光探针, 采用时间扫描方式记录血小板内 Ca^{2+} 的变化; 分别用 HPLC/ECD 和放射免疫测定法检测 5-HT 及 TXB_2. **结果**: MN-9202 剂量依赖地抑制 ADP 或凝血酶诱导的血小板聚集, 抑制 TXA_2 的释放并且能有效阻滞激活血小板胞内 Ca^{2+} 水平的增加. MN-9202 1 $\mu mol \cdot L^{-1}$ 能抑制胶原 15 $mg \cdot L^{-1}$ 诱导的 5-HT 释放反应, 但对胶原 45 $mg \cdot L^{-1}$ 诱导的反应无抑制作用. **结论**: MN-9202 阻滞血小板 Ca^{2+} 内流并抑制血小板花生四烯酸代谢及激活反应.

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