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Post-stroke atorvastatin treatment reduces neurological deficits and mortality rate in the stroke-prone spontaneously hypertensive rat

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Abstract

Several large clinical trials have demonstrated that 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitors decreased the incidence of stroke independently of their cholesterol-lowering effect. We have investigated the effect of post-stroke treatment with atorvastatin on neurological deficits and mortality in stroke-prone spontaneously hypertensive rats (SHR-SP). The vehicle-treated group showed significantly aggravated neurological deficits compared with those observed on the first day of stroke. In contrast, the post-stroke oral administration of atorvastatin at 3 or 30 mg kg^{-1}/day significantly ameliorated these neurological deficits. Atorvastatin improved the survival rate in a dose-dependent manner, with this effect being significant at 30 mg kg^{-1}/day. Atorvastatin did not affect blood pressure, heart rate or total cholesterol in SHR-SP at either dose. In contrast, it significantly increased plasma nitric oxide (NO) levels at both doses. These results indicated that post-stroke administration of atorvastatin ameliorated neurological deficits and prolonged survival, which might have resulted from increased plasma NO, apart from its effect on cholesterol level and blood pressure in SHR-SP. In conclusion, this study demonstrated the protective effects of post-stroke administration of atorvastatin against stroke in SHR-SP.

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Introduction

The best-known action of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) is to decrease the biosynthesis of cholesterol by blocking mevalonate synthesis. In addition to this cholesterol lowering action, a growing body of clinical and experimental evidence has suggested that statins exhibit additional beneficial actions beyond cholesterol reduction. Several large clinical trials (Pedersen 1994; Sacks et al 1996) demonstrated that statins decreased the incidence of stroke and myocardial infarction, and suggested that these agents exerted beneficial effects due to the restoration of endothelial function independently of their cholesterol lowering effect (Delanty & Vaughan 1997). In animal ischaemia models, mainly produced by acute occlusion of the cerebral artery, statins have been reported to upregulate endothelial nitric oxide synthase (eNOS), reduce the size of infarction and exert neuroprotective action (Endres et al 1998; Amin-Hanjani et al 2001).

Hypertension is a major risk factor for stroke. The dysfunction and injury of cerebral arteries following stroke caused by acute occlusion of cerebral arteries, however, is not identical to that by long-term hypertension. Stroke-prone spontaneously hypertensive rats (SHR-SP) develop severe hypertension from an early age, which results in stroke (Tagami et al 1987), and have therefore been widely used in assessing pharmacological agents that are putatively capable of protection against stroke (Ogiku et al 1993; Biagini et al 1997).

Atorvastatin, a currently available statin, is reported to decrease the size of infarction in a murine acute cerebral ischaemia model produced by cerebral artery occlusion (Namura et al 2001; Sasamata et al 2002). However, the effect of atorvastatin on stroke resulting from known risk factors such as hypertension is unknown. Furthermore, the effects of statins on stroke in previous studies were largely determined by pre-stroke treatment

(Amin-Hanjani et al 2001; Balduini et al 2001; Kawashima et al 2003). The purpose of this study, therefore, was to investigate the effect of post-stroke treatment with atorvastatin on neurological deficits and mortality in SHR-SP.

Materials and Methods

Drugs

Atorvastatin (Atorvastatin calcium, Lot 1A0511) was provided by Pfizer Pharmaceuticals (Ann Arbor, MI). It was suspended in aqueous 0.5% methylcellulose at concentrations designed to provide the appropriate dose at $5\,mL\,kg^{-1}$ and administered at doses of 3 or $30\,mg\,kg^{-1}$/day by single daily oral gavage. Doses of atorvastatin were selected by reference to previous reports (Namura et al 2001; Sasamata et al 2002).

Determination of neurological deficits and survival rate

Adult male SHR-SP (SHR-SP/Izm; 9-weeks old; Japan SLC, Inc.) were housed in a room maintained at $21 \pm 1°C$ with a 12-h light cycle (lights on 0700 h) and given free access to a special food, SP-feed (Funabashinoujyou, Inc.), and tap water.

The rats were weighed and monitored daily for behavioural signs of stroke (Smeda 1989; Smeda et al 1999). Stroke onset in this strain is always accompanied by pathognomonic neurological deficits such as hemiplegia and seizure (Yamamoto et al 1991). The first observation of neurological deficits was defined as positive evidence of the first stroke. The first stroke in this model is accompanied by abrupt weight loss of more than 5 g, which may be due to the inability to access food or water when the stroke occurs (Yamamoto et al 1988, 1989, 1991). After the first stroke, rats in the study were randomly assigned to one of three groups, namely vehicle or atorvastatin at 3 or $30\,mg\,kg^{-1}$/day group ($n = 33$ for each vehicle-treated group and atorvastatin-treated group). Vehicle or atorvastatin (3 or $30\,mg\,kg^{-1}$) was orally administered once a day from day of the first stroke to death.

A blinded observer evaluated the neurological deficits on the day the first stroke occurred, and 7 and 14 days later, by the method of Yamamoto et al (1988, 1989, 1991) with modification. Paralysis of the right and left forelimb, right and left hindlimb, and lethargy were examined as parameters of neurological deficits. These parameters were classified into 0–3 grades according to the following definition: score 0, normal; score 1, mild deficit; score 2, moderate deficit; score 3, severe deficit. Final score per animal was evaluated by adding the score of each parameter for a total maximum score of 15.

Measurement of blood pressure, plasma total cholesterol and nitric oxide

Blood pressure, plasma total cholesterol, and nitric oxide (NO) were evaluated 3 h after the final administration, namely on day 14 of administration. Blood pressure and heart rate were evaluated by the tail-cuff method using Softron BP 98-A equipment (Softron Co. Ltd) under conscious conditions ($n = 8$ for each vehicle-treated group and atorvastatin-treated group). For plasma total cholesterol and NO measurement, blood was collected from the inferior vena cava of ether-anaesthetized rats ($n = 6$ for each vehicle-treated group and atorvastatin-treated group). All samples were centrifuged ($3000\,rev\,min^{-1}$, 10 min) in a high-speed cooler centrifuge (Hitachi Himac CR5DL; Hitachi Co. Ltd) and the plasma was used to measure total cholesterol and NO. The amount of total cholesterol in the plasma was measured with an enzymatic lipid assay kit, Cholesterol C-test Wako (Osaka, Japan), and a spectrophotometer (U-3210; Hitachi Co. Ltd). To measure plasma NO, the plasma was mixed with the same amount of methanol and centrifuged ($3000\,rev\,min^{-1}$, 10 min) to deproteinize the plasma. The amount of NO_2^- and NO_3^- contained in the deproteinized plasma was measured by a NO analyser (ENO-200 series, EiCOM, Kyoto, Japan) (Tabuchi et al 2002). Total plasma NO was determined by the addition of NO_2^- and NO_3^- contents.

Statistics

Data for body weight, systolic blood pressure, diastolic blood pressure, mean blood pressure, heart rate, total cholesterol and NO are expressed as mean ± s.e.m. Statistical differences for these parameters were analysed using one-way analysis of variance followed by Dunnett's multiple range test. Survival rate was tested by the log-rank test. Neurological deficits was tested by Kruskal–Wallis test followed by Mann–Whitney U-test for the individual difference, then adjusted P value by Bonferroni's method. P values less than 0.05 were considered statistically significant.

Results

Effects of atorvastatin on neurological deficits

From an initial 99 SHR-SP, 11 (11.1%) animals died before administration and seven (7.0%) did not develop stroke before 14-weeks old, and so were excluded from the experiment. The remaining rats (81.8%) developed stroke from 10- to 13-weeks old and were used for the experiments. They exhibited signs of stroke such as paralysis of limbs, lethargy and savagery, accompanied by abrupt weight loss. Vehicle or drug administration was started after the onset of the first stroke. The vehicle-treated group showed significantly aggravated neurological deficits at two weeks after the first stroke compared with those on the day of the first stroke. Atorvastatin (3 or $30\,mg\,kg^{-1}$/day, p.o.) tended to ameliorate the observed neurological deficits after administration for one week (Figure 1). Significant amelioration of neurological deficits was observed after administration of atorvastatin 3 or $30\,mg\,kg^{-1}$/day for two weeks, with amelioration to almost normal behaviour seen with $30\,mg\,kg^{-1}$/day.

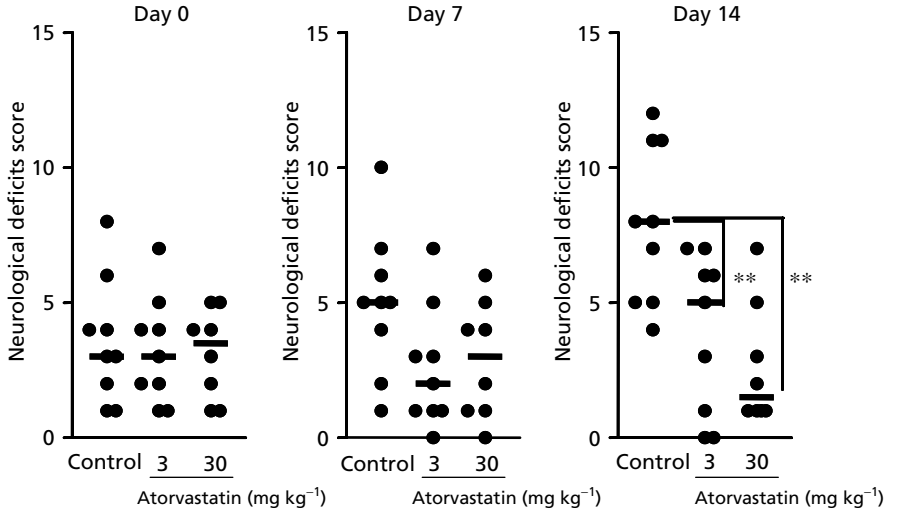

Figure 1 Effect of post-stroke administration of atorvastatin on neurological deficits in SHR-SP. Neurological deficit score was observed on day 0, the day that the first stroke occurred, and on days 7 and 14. Single daily oral administration of atorvastatin at 3 or 30 mg kg^{-1} was started on day 0. Dots represent individual scores and bars represent the median. Asterisks indicate significant differences to control values by Kruskal–Wallis test followed by Mann–Whitney U-test for the individual difference, then adjusted P value by Bonferroni's method (**$P < 0.01$).

Effects of atorvastatin on survival period

The vehicle-treated group showed a survival rate of 3/28 (10.7%) at five weeks after the first stroke. Atorvastatin at 3 or 30 mg kg^{-1}/day dose-dependently improved survival at this time to 6/29 (20.7%) and 8/24 (33.3%), respectively (Figure 2). Improvement was significant at 30 mg kg^{-1}/day by the log-rank test.

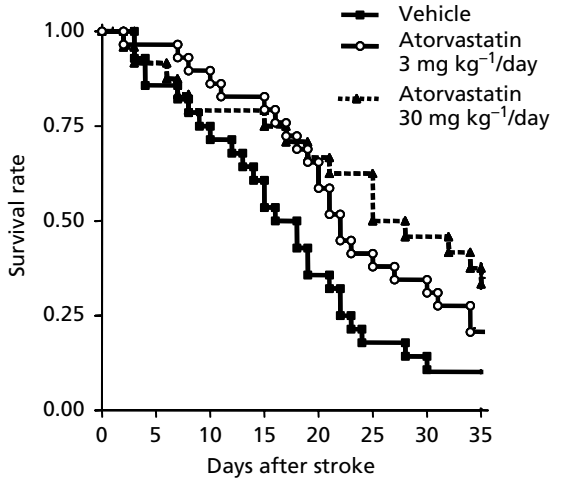

Figure 2 Effect of post-stroke administration of atorvastatin on survival rate in SHR-SP. Single daily oral administration of atorvastatin at 3 or 30 mg kg^{-1} for 14 days was started on day 0, the day that the first stroke occurred. Atorvastatin improved survival rate in a dose-dependent manner, significantly so at 30 mg kg^{-1} by the log-rank test ($P < 0.05$).

Effects of atorvastatin on blood pressure and lipid profile

On analysis of physiological parameters and blood pressure, severe hypertension (mean blood pressure 185 mmHg) was seen in SHR-SP at 12-weeks old. Atorvastatin (3 or 30 mg kg^{-1}/day, p.o.) did not affect body weight, blood pressure, heart rate or total cholesterol in SHR-SP after administration for two weeks (Table 1).

Effects of atorvastatin on plasma NO

NO was 12.1 ± 0.5 nmol mL^{-1} in SHR-SP at age 12 weeks. Atorvastatin significantly increased plasma NO in a dose-dependent manner, with levels of 16.8 ± 1.0 and 21.2 ± 1.4 nmol mL^{-1} at 3 and 30 mg kg^{-1}/day, respectively (Figure 3).

Discussion

These results showed that post-stroke administration of atorvastatin prolonged survival and ameliorated neurological deficits in SHR-SP. The use of this strain allowed us to determine the effect of atorvastatin on hypertension-based incidental stroke and associated symptoms. The nature of stroke in SHR-SP is more complex than that in the cerebral artery occlusion model: SHR and SHR-SP show a decrease in cerebral blood flow during the development of hypertension (Tamaki et al 1995; Yamori & Horie 1977), and thus the aetiology of stroke in these rats is closely similar with that in ischaemic patients (Yamori et al 1976; Akiguchi et al 1977). In SHR-SP, stroke is reported to begin as very minor cerebral lesions, typically

Table 1 Effect of atorvastatin on blood pressure and plasma total cholesterol

Parameter	Vehicle	Atorvastatin 3 mg kg^{-1}	Atorvastatin 30 mg kg^{-1}
Body weight (g)	253 ± 3	258 ± 3	250 ± 5
Systolic blood pressure (mmHg)	214 ± 6	212 ± 7	203 ± 8
Diastolic blood pressure (mmHg)	170 ± 7	164 ± 8	156 ± 5
Mean blood pressure (mmHg)	185 ± 6	180 ± 7	172 ± 6
Heart rate (beats min^{-1})	431 ± 20	414 ± 30	373 ± 19
Total cholesterol (mg dL^{-1})	78.6 ± 2.2	84.3 ± 4.3	67.9 ± 4.6

Atorvastatin, administrated once a day at 3 or 30 mg kg^{-1} orally for two weeks, had no effect on blood pressure and plasma total cholesterol. Parameters were measured and samples were collected on the last day of drug administration. All values represent the mean ± s.e.m. Significance of differences was tested by analysis of variance followed by Dunnett's multiple range test.

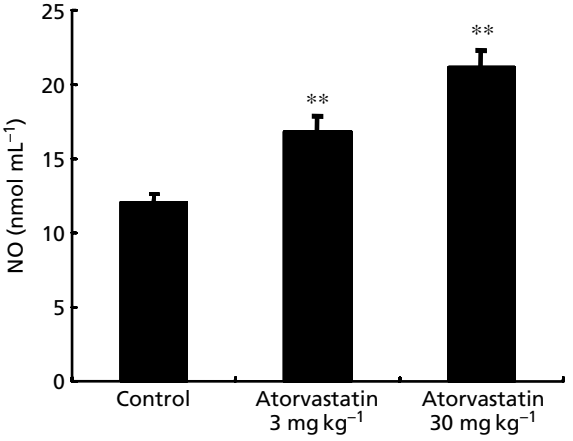

Figure 3 Effect of atorvastatin on plasma NO. Single daily oral administration of atorvastatin at 3 or 30 mg kg^{-1} for 14 days was started on day 0, the day that the first stroke occurred. Samples were collected on the last day of drug administration. Atorvastatin dose-dependently and significantly increased plasma NO. All values represent the mean ± s.e.m. Asterisks indicate significant differences to vehicle values by analysis of variance followed by Dunnett's multiple range test (**$P < 0.01$).

consisting of small, pinpoint-sized haemorrhage (Smeda 1989), followed several weeks later by multiple cerebral haemorrhage or infarct and severe neurological deficit (Kawashima et al 2003). In our study, neurological deficit scores in the vehicle-treated group were aggravated following the first stroke. The stroke was accompanied by abrupt weight loss and neurological deficits including hemiplegia and seizure, as reported by Yamamoto et al (1991). Atorvastatin (3 or 30 mg kg^{-1}/day) significantly inhibited this aggravation of neurological deficits following the first stroke (Figure 1).

The amelioration of neurological deficits may have resulted from inhibition of the further development of stroke and recurrent stroke. In previous reports, atorvastatin and other statins have shown neuroprotective effects on pre-stroke treatment (Amin-Hanjani et al 2001; Balduini et al 2001; Kawashima et al 2003). In this study, post-stroke treatment with atorvastatin ameliorated neurological deficits and decreased mortality. The median survival days of control, atorvastatin 3 and 30 mg kg^{-1} groups was 17, 22 and 27 days, respectively. Given this, atorvastatin may have a secondary preventive action in stroke patients.

To determine which parameters contributed to the amelioration of neurological deficits and the decrease in the mortality rate, parameters such as blood pressure and total cholesterol were measured after atorvastatin administration. Severe hypertension was observed, with blood pressure values similar to those previously reported in SHR-SP (Kawashima et al 2003). Administration of atorvastatin did not affect blood pressure or total cholesterol level, indicating that the effects of atorvastatin on blood pressure or total cholesterol were not involved in the amelioration of neurological deficits and decrease in mortality in this study. Although in this study atorvastatin did not affect blood pressure, Kishi et al (2003) reported that atorvastatin (50 mg kg^{-1}/day for 30 days) decreased systolic blood pressure, which was reflected in our pilot studies. This discrepancy may be due to the difference in dosage in the experiments. In this study, we stated the maximum dose as 30 mg kg^{-1}/day to see the effect of atorvastatin without its blood pressure lowering effect.

Interestingly, plasma NO was significantly increased by atorvastatin at 3 or 30 mg kg^{-1}/day. NO is an important mediator of vascular homeostasis and blood flow (Furchgott & Zawadzki 1980; Palmer et al 1987; Ignarro 1990; Radomski et al 1990; Huang et al 1995). It has been reported in animal ischaemia models that inhibition of endothelial NO activity decreased cerebral blood flow and promoted tissue damage after focal ischaemia (Huang et al 1994), and that enhancement of NO production by either administration of NO donors or eNOS substrate conferred stroke protection after induction of cerebral ischaemia (Morikawa et al 1992; Dalkara et al 1994; Zhang & Iadecola 1994). Many studies have implicated NO in the pathogenesis of hypertension and stroke in SHR-SP. Onda et al (1994) demonstrated that the aortic endothelium in SHR-SP produced low amounts of NO

compared with those in WKY, and Malinski et al (1993) demonstrated decreased NO synthase activity in SHR-SP compared with WKY. Noguchi et al (1999) reported that constant intake of NO donor decreased the incidence of stroke in SHR-SP while administration of NO synthase inhibitor increased it.

The importance of NO has been reported in man. In clinical studies, stroke patients had lower levels of NO compared with controls, and patients who died or became institutionalized had lower NO than patients who were discharged home (Rashid et al 2003). These findings suggested that the increase in NO by post-stroke administration of atorvastatin was the key factor in the amelioration of neurological deficits. Laufs et al (2000) demonstrated that atorvastatin showed a neuroprotective effect due to its activation of eNOS. Other statins have also been reported to increase eNOS activity (Endres et al 1998; Laufs et al 1998; Amin-Hanjani et al 2001). In this study, therefore, the increase in NO by atorvastatin may be attributed to the activation of eNOS in the endothelium.

Atorvastatin at $30\,mg\,kg^{-1}$/day significantly improved survival time in SHR-SP. Stroke in this strain is characterized by symptoms similar to those seen in stroke patients. Pathology studies of clinical stroke have indicated that the inflammatory process is involved in its development (Emsley & Tyrrell 2002). An elevated C-reactive protein (CRP) level, which indicates low-grade inflammation, is suggested to be a risk factor for the development of ischaemic stroke and transient ischaemic attack (Ridker et al 1997; Rost et al 2001). Statins are reported to lower CRP levels in a manner largely independent of effects on low-density lipoprotein cholesterol (Albert et al 2001). In addition, statins are reported to demonstrate anti-inflammatory effects by upregulating cytokine interleukin-6, which controls CRP (Yokota et al 2003). Taken together, these reports suggest that an effect on the inflammatory system is involved in the beneficial effects of atorvastatin.

Conclusion

We have demonstrated that the post-stroke administration of atorvastatin conferred protective effects against stroke in SHR-SP. These effects may derive from NO activation, as well as its effect on blood pressure and total cholesterol.

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