BIPHASIC EFFECTS OF ATORVASTATIN ON INFLAMMATION

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ABSTRACT

Statins have been shown to exert 'pleiotropic effects' independent of their cholesterol lowering actions that include anti-inflammatory properties. We show in this study that atorvastatin dependent on the way of administration may exert anti- or pro- inflammation effects. Carrageenan-induced rat paw edema and mouse air-pouch as inflammatory models were used in this study. Animals were received statins orally prior to induction of inflammation by injection of carrageenan into rat paw or the pouch. The local effect of atorvastatin was determined by injection of the drug into the pouch. Oral administration of statins reduced both the maximal edema response and neutrophils infiltration in the inflammation zone. Lovastatin had the lowest and atorvastatin had the greatest effects. Also, in the mouse air-pouch model oral treatment by atorvastatin produced a very significant ($p<0.0001$) reduction in carrageenan-induced pouch leukocyte recruitment and exudates production. Concurrent administration of mevalonate reversed the anti-inflammatory effect of atorvastatin. However, local injection of atorvastatin into the pouch induced a dose depend and significant increase in leukocyte recruitment into the pouch that was not reversed by co-administration of mevalonate. This study shows that atorvastatin dependent on the way of administration has both pro- and anti- inflammatory properties. Contrary to anti-inflammatory effects, the pro-inflammatory responses are independent of HMG CoA reductase inhibition and can be mediated directly by atorvastatin.

Keywords: Statins, atorvastatin, anti-inflammatory, pro-inflammatory.

INTRODUCTION

Hyperlipidemia is a major cause of atherosclerosis and atherosclerosis-associated conditions such as coronary heart disease (CHD), ischemic cerebrovascular disease, and peripheral vascular disease. Many well-controlled clinical trials established the benefits of 3-hydroxy -3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) on fatal and nonfatal CHD events and strokes (Scandinavian Simvastatin Survival Study, 1994; Shepherd *et al*., 1995; The Long-Term Intervention with Pravastatin, 1998; Heart Protection Study Collaborative Group, 2002; Heart Protection Study Collaborative Group, 2003). The observation of that statins reduce the risk of cardiovascular events even in the absence of a significant decrease of blood cholesterol levels, supports the relevance of the potential "pleiotropic" functions of this drug class (Downs *et al*., 1998; Ridker *et al*., 1998). A large number of studies reported a prominent role of inflammation and immune response on the development of atherosclerotic plaques and their destabilization (Steinberg, 2005; Arnaud *et al*., 2005; Stoll and Bendszus , 2006). Approximately, all in vitro and in vivo studies uniformly support anti-inflammatory roles of statins. Anti-inflammatory properties of statins probably include various mechanisms that may or may not involve the HMG-CoA reductase/mevalonate pathway (Schonbeck and Libby, 2004). However, a recent report highlights the pro-inflammatory effects of statins in mitogen-activated peripheral blood mononuclear cells through the activation of caspase-1 and IL-18 secretion in monocytes (Coward *et al*., 2006).

In this study we showed that oral administration of atorvastatin, simvastatin, and lovastatin have lipid-lowering-independent anti-inflammatory and anti-leukocyte accumulation activities in carrageenan induced rat paw edema model. The potency and effectiveness of statins in this model was comparable to their inhibitory potency on HMG-CoA reductase. It has been shown that mevalonic acid co-treatment reverses anti-inflammatory and anti-neutrophils accumulation effects of statins (Diomede *et al*., 2001; Fessler *et al*., 2005), indicating the role of nonsterol mevalonic acid pathways in the anti-inflammatory actions of statins. However, it remains to be determined whether statins have direct interference with inflammatory pathways rather than inhibition of HMG-CoA reductase. The aim of this study is to evaluate the anti- or pro- inflammatory effects of atorvastatin in carrageenan-induced rat paw edema or in air-pouch model of local inflammation when used orally or injected into the pouch.

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MATERIALS AND METHODS

Reagents

Atorvastatin was kind gift from Sobhan Pharmaceutical Inc (Tehran-Iran). Lovastatin and Simvastatin were provided by Arya and Shahr Daru Pharmaceutical companies (Tehran-Iran), respectively. Carrageenan was from Sigma (Germany). All other reagents were of analytical grade.

Experimental animals

Male Wistar rats (200-250 g; n=6-8) and male mice (20-22 g; n=6-8) were used in this study. The animals were given food and water ad libitum. They were housed in the Animal House of Tabriz University of Medical Sciences at a controlled ambient temperature of 25 ± 2°C with 50 ± 10% relative humidity and with a 12-h light/12-h dark cycle (lights on at 7:00 a.m.). This study was performed in accordance with the Guide for the Care and Use of Laboratory Animals of Research affairs of Tabriz University of Medical Sciences, Tabriz-Iran.

Carrageenan-induced paw edema

Carrageenan-induced rat paw edema was used as an acute inflammation model. Male wistar rats received a subplantar injection in the right hind-paw of 100µl of 1% carrageenan in saline. Footpad volume was measured by a plethysmometer (UGO BASILE 7140, Italy) prior to carrageenan injection and then at hourly intervals from 1-4h afterwards (Maleki *et al*., 2001). Data are expressed as percent increase in paw volume (in microliters) compared to the pre-injection values. The inflammatory response in the drug-treated and control was also measured as the area under the time-course curves (AUC). Rats received carboxymethylcellulose (CMC 0.5%; control), indo-methacin (2.5 mg/kg; positive control), or 1, 5, and 10 mg/kg of statins in 0.5% CMC orally 20, 12, 6, and 1h prior to inflammation induction.

Histological examination

Histological studies were carried out to determine leukocytes accumulation and the relationship between microscopic structure of paw tissue and inflammation induced by carrageenan in paw. Biopsies of paws were taken and the tissue slices were fixed in 10% neutral-buffered formaldehyde, embedded in paraffin, and sectioned. The sections were stained with haematoxylin and eosin and studied by light microscopy. The number of polymorphonuclear leukocytes (PMN) was counted in five random high-power fields in each sample by a light microscope.

Air-pouch model of inflammation

Subcutaneous dorsal pouches were created in mice by injection of 5 mL of air and by reinjection, 3 days later, of 3 mL of air (Diomede *et al*., 2001). On day 6, 1 mL of 1% carrageenan was injected into the pouches. The inflammation was evaluated by determination of the volume of exudates and the number of leukocytes recruited into the pouch (using Sysmex KX-21 cell counter) 24 hours after induction of inflammation. To asses the systemic effect of atorvastatin on inflammation, 10 mg/kg atorvastatin in 0.5% CMC was given by gavages to pouch-bearing mice 20, 12, 6, and 1 hour before carrageenan. The local effect of 10, 25, 50, and 75 µg atorvastatin and 50 µg indomethacin dissolved in 0.5 ml DMSO (%1 in saline) was determined by injection of the drug into the pouch 1 hour before and 8 and 20 hours after carrageenan injection into the pouch. We also tested whether intraperitoneal injection of 10 mg/kg mevalonate in saline or intra-pouch injection of 50µg mevalonate in DMSO (%1) reversed the effect of atorvastatin.

Cholesterol and triglyceride assay

At the end of experiments rats were anaesthetized by intra peritoneal (i.p) injection of pentobarbital (60mg/kg) and blood were collected from Jugular vein. Serum total cholesterol and triglyceride were assayed by a standard enzymatic method of Watson (1960), Fossati and Principe (1982) respectively.

STATISTICS

Data were presented as mean ± SD. Comparisons between groups were made with Student's t test or ordinary ANOVA as appropriate. If ANOVA analysis indicated significant differences, a Student-Newman-Keuls post test was performed to compare mean values between treatment groups and control. Differences between groups were considered significant at $p<0.05$.

RESULTS

Effects of statins on carrageenan-induced paw edema

Subplantar injection of carrageenan into the paw is followed by swelling of the footpad that can be reproducibly measured after 4 hours. Treatment with indomethacin blocked swelling markedly (table 1). We found that oral admistration of all three statins, atorvastatin, simvastatin, and lovastatin 20, 12, 6, and 1h prior to induction of inflammation reduced both the maximal oedema response attained during 4h and neutrophils infiltration into the inflammation zone (fig 1). Lovastatin had the lowest and atorvastatin had the greatest effects (table 1, fig. 1). The mean total edema responses (AUC) were also reduced significantly by 10 mg/kg of all statins. The strongest inhibitory effect on the total edema response which was comparable to that of indomethacin was seen by atorvastatin and then by simvastatin (fig 2). These observations strongly suggest that oral treatment by statins has anti-inflammatory activities. The footpad swelling represents an acute inflammatory response characterized by the influx of PMN leukocytes. Statins blocked the influx of PMN leukocytes into the paw 4

Table 1: Effects of Statins (orally) and Indomethacin (positive control) on carrageenan-induced paw edema in rats compared to control group (vehicle). Drugs were given orally 20, 12, 6, and 1 hours before carrageenan injection into the paws.

Group	Increase in paw thickness (%)			
	1h	2h	3h	4h
Control (carageenan)	26±2	47±5	69±7	89±6
Indometacin (2.5mg/kg)	9±2***	15±1***	24±2***	24±1***
Lovastatin (1 mg/kg)	22±1	37±3	78±5	99±3
Lovastatin (5 mg/kg)	15±3***	29±2***	76±4	97±3
Lovastatin (10 mg.kg)	11±3***	24±3***	73±3	93±6
Simvastatin (1 mg/kg)	16±2***	36±3**	66±3	59±2***
Simvastatin (5 mg/kg)	15±2***	28±1***	62±4**	54±1***
Simvastatin (10 mg.kg)	15±1***	27±3***	60±2**	50±3***
Atorvastatin (1 mg/kg)	12±3***	30±4***	50±6***	52±4***
Atorvastatin (5 mg/kg)	7±2***	15±1***	32±2***	37±2***
Atorvastatin (10 mg.kg)	8±1***	14±2***	25±3***	21±1***

***p<0.01 and ***p<0.001* compared to the same points in the control group using one-way ANOVA and Student-Newman-Keuls post-test

Table 2: Effects of atorvastatin in the presence and absence of mevalonate on paw edema and on PMN accumulation in the inflammation zone. Atorvastatin was given orally 10 mg/kg and mevalonated was used i.p (10mg/kg). Drugs were given 20, 12, 6, and 1 hours before carrageenan injection into the paws. The number of PMN was counted in five random high-power fields in each sample (n=6) by a light microscope.

Group	Increase in paw thickness (%)				PMN number
	1h	2h	3h	4h	
Control	28±2	49±4	69±6	90±9	24±2
Atorvastatin	10±5	18±5***	26±7**	45±2***	10±0.8***
Mevalonate	25±4	43±6	71±6	96±6	28±1
Ator + mevalonate	12±3	33±6	68±8	94±11	24±2

***p<0.01 and ***p<0.001* compared to the same points in control, mevalonate and atorvastatin + mevalonate groups using one-way ANOVA and Student-Newman-Keuls post-test

hours after carrageenan injection (fig 1). As shown in the fig. 1, 1mg/kg lovastatin and simvastatin did not affect the ability of leukocytes to migrate at the inflammation site, whereas the dose of 1 mg/kg of atorvastatin reduced the number of total leukocytes recruited by carrageenan very significantly ($p<0.0001$). Maximal suppression by atorvastatin was comparable to that of indomethacin. Plasma lipids were measured in samples taken at the end of the experiments for all groups. The statins did not alter plasma cholesterol and triglycerides (data not shown). To determine the role of mevalonate pathway in anti-inflammatory effect of statins, rats were given 10 mg/kg atorvastatin (PO) and concurrently mevalonate (10 mg/kg; IP) 20, 12, and 1 hours before carrageenan injection into the paws. As shown in table 2 mevalonate alone did not affect the paw edema or PMN accumulation in the inflammation site, whereas, when given with atorvastatin, it completely reversed the anti-inflammatory effects of atorvastatin.

Effects of Atorvastatin on air-pouch model of inflammation

The anti-inflammatory action of oral administration of atorvastatin, 20, 12, 6 and 1 hours prior to induction of inflammation, was then evaluated by determination of its effect on the exudates volume and the number of leukocytes recruited into the pouch in air-pouch model of local inflammation. As shown in fig. 3, atorvastatin (10 mg/kg) orally reduced the leukocyte recruitment and exudates volume very considerably. Whereas, concurrent i.p injection of mevalonate (10 mg/kg) reversed the anti-inflammatory effects of atorvastatin.

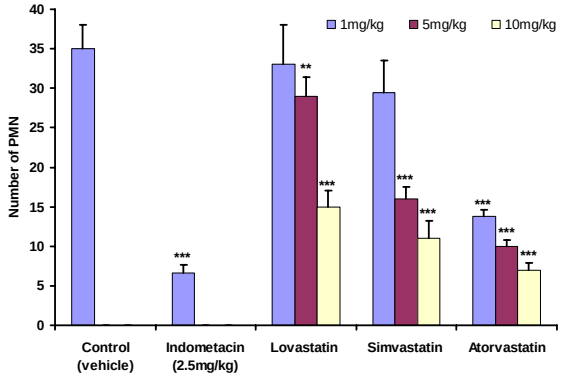

Fig. 1: Effects of statins (orally) on leukocyte recruitment into the inflammation site. The number of PMN was counted in five random high-power fields in each sample (n=6) by a light microscope. Controls received the corresponding vehicle alone. Rats in positive control group were treated by 2.5 mg/kg indomethacin. Indometachin or statins (1, 5, and10 mg/kg) was given orally to rats 20, 12, 6, and 1 hour before carrageenan injection. ***P*< 0.001 and ****P*<0.0001 vs control, using Student's t test.

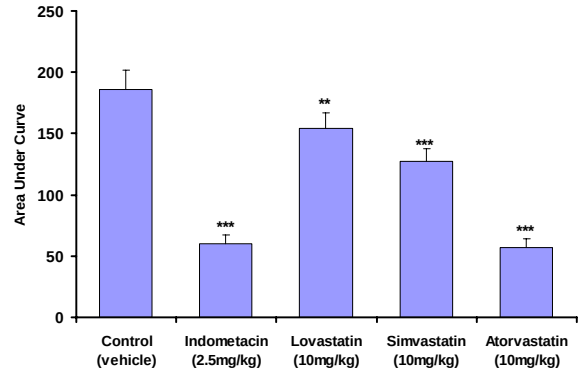

Fig. 2: Effects of statins (orally) on total edema response measured as Area Under Curve (AUC) of carrageenan-induced paw edema response in rats during 4 hr. Controls received the corresponding vehicle alone. Rats in positive control group were treated by 2.5 mg/kg indomethacin. Indometachin or statins (10 mg/kg) was given orally to rats 20, 12, 6, and 1 hour before carrageenan injection. ***P*< 0.001 and ****P*<0.0001 vs control, using Student's t test.

Fig. 4 shows the effect of local injection of atorvastatin in different doses into the pouch in pouch bearing mice. Atorvastatin in a dose depend manner increased the number of leukocytes recruited into the pouch by carrageenan. There was very marked (p<0.0001) elevation in the total number of leukocyte accumulated in the inflammation site by 50 and 75 µg of atorvastatin. However, the exudates volume was not affect by atorvastatin even it was reduced significantly (p<0.001) by 10 µg of the drug. Local administration of indomethacin (50 µg) in this model produced a significant (p<0.0001) reduction both in leukocyte number and exudates volume. The effect of intra-pouch injection of mevalonate (50 µg) was the same as the control group and had no effect on pro-inflammatory action of 50 µg of atorvastatin.

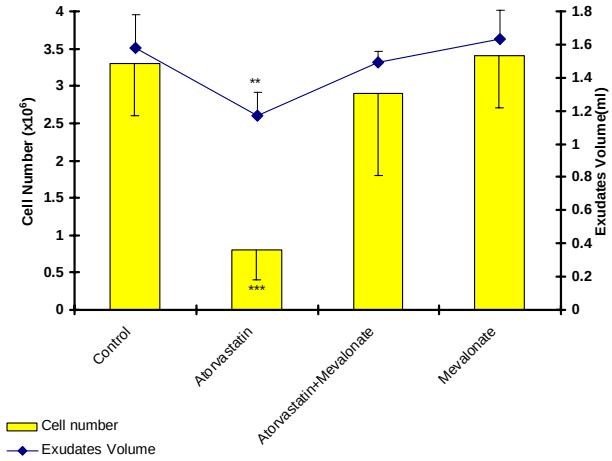

Fig. 3: Effect of oral administration of atorvastatin (10mg/kg) on leukocyte recruitment (Total leukocytes) and pouch exudates volume (ml) in air-pouch model. Controls received the corresponding vehicle alone. Atorvastatin (10 mg/kg) and mevalonate (10 mg/kg) were given orally and intraperitoneally, respectively, to mice 20, 12, 6, and 1 hours before carrageenan injection into the pouch. ***P*< 0.001 and ****P*<0.0001 vs control, using one-way ANOVA and Student-Newman-Keuls post-test.

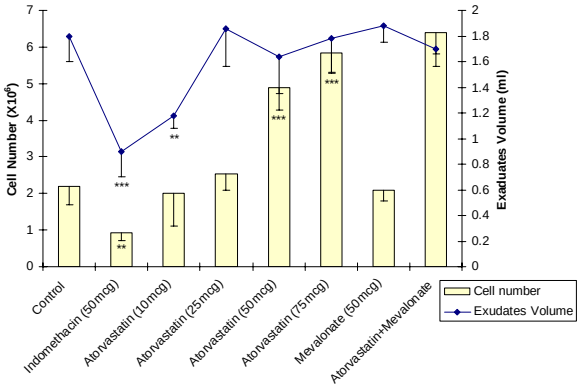

Fig. 4: Effect of local administration of atorvastatin (10, 25, 50 and 75 µg/kg) or indomethacin (50 µg/kg) on leukocyte recruitment (Total leukocytes) and pouch exudates volume (ml) in air-pouch model. Controls received 0.5 ml DMSO (%1 in saline; vehicle) alone. Atorvastatin and mevalonate (50 µg/kg) were injected into the pouch in vehicle 1 hour before and 8 and 20 hours after carrageenan injection into the pouch. ***P*< 0.001 and ****P*<0.0001 vs control, using one-way ANOVA and Student-Newman-Keuls post-test.

DISCUSSION

In this study we showed that oral administration of atorvastatin, simvastatin, and lovastatin have lipid-lowering-independent anti-inflammatory and anti-leukocyte accumulation activities in carrageenan induced rat paw edema model. The potency and effectiveness of statins in this model was comparable to their inhibitory potency on HMG-CoA reductase. Where, atorvastatin had the strongest and lovastatin the weakest anti-inflammatory effect. Furthermore, the anti-inflammatory effect of atorvastatin was reversed by mevalonate, suggesting the involvement of mevalonate pathway in these effects of statins. We also demonstrated that atorvastatin induced dose-dependent pro-inflammatory effect in air-pouch inflammation model when injected into the pouch. Concurrent administration of mevalonate along with atorvastatin failed to inhibit the pro-inflammatory action of the drug.

Statins differ in their pharmacokinetics properties. Under steady state conditions, small amounts of the parent drug can be found in the systemic circulation. Serum level of atorvastatin ranged between 2 and 200 μg/l, which is prescribed at doses of 10-80 mg/day (Cilla *et al*., 1996). However, such small concentration of statins, especially potent statins like atorvastatin with a long plasma half life of 20 hours, may demonstrate significant biological effects.

In summary, atorvastatin has a biphasic pro- and anti-inflammatory effects that are mainly independent of its effects on blood cholesterol and the anti-inflammatory effects are related to mevalonic acid pathway. Atorvastatin may exert its pro-inflammatory effect directly via a mevalonat independent pathway. These results suggest that extra hepatic and direct effects of statins might play a potentially undesirable role in patients who receive potent and long acting statins for a long time.

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